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Science

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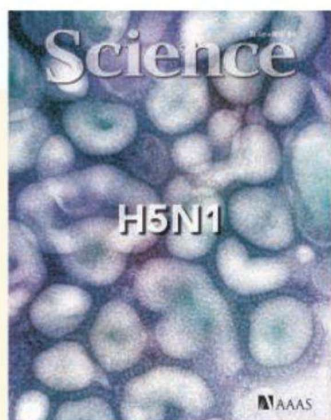
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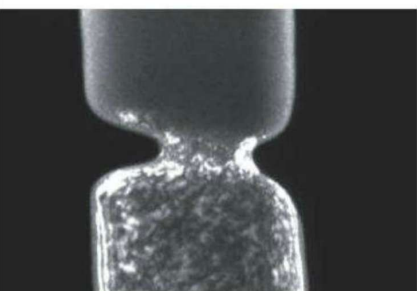
Image: Dr. Gopal Murti/Visuals Unlimited, Inc.

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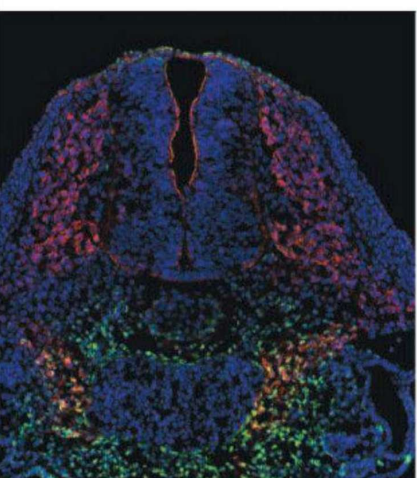
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2.8 Million Years of Arctic Climate Change from Lake El'gygytyn, NE Russia

M. Melles et al.

A sediment core from a Russian lake provides a high-latitude climate record where prior terrestrial records have been sparse.

10.1126/science.1222135

Kepler-36: A Pair of Planets with Neighboring Orbits and Dissimilar Densities

J. A. Carter et al.

The Kepler spacecraft detected a super-Earth and a Neptune-like planet in very tightly spaced orbits around the same star.

10.1126/science.1223269

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S. Aral and D. Walker

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10.1126/science.1215842

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Human α -Defensin 6 Promotes Mucosal Innate Immunity Through Self-Assembled Peptide Nanonets

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Rather than killing bacteria directly, a gut antimicrobial peptide forms netlike structures that ensnare invading bacteria.

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M. D. Van Kerkhove et al.

Full text at www.sciencemag.org/cgi/content/full/336/6088/1506-b

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T. T. Wang and P. Palese

Full text at www.sciencemag.org/cgi/content/full/336/6088/1506-c

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Computer Program 'Evolves' Music From Noise
DarwinTunes helps explain how composers refine their compositions based on audience input.

http://scim.ag/DarwinTunes_Composers

Stem Cells Move Into Prime Time

Two promising studies head toward clinical research.

http://scim.ag/Promising_Studies

You Owe Your Life to Rock

The erosion of metal-rich granite long ago set the stage for multicellular organisms.

http://scim.ag/Life_Rock

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19 June issue: <http://scim.ag/ss061912>

RESEARCH ARTICLE: Direct Modification and Activation of a Nuclear Receptor-PIP₂ Complex by the Inositol Lipid Kinase IPMK

R. D. Blind et al.

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H. A. Ingraham et al.

The transcriptional activity of a nuclear receptor is regulated by the phosphorylation status of a bound lipid.

RESEARCH ARTICLE: Histone Deacetylases 6 and 9 and Sirtuin-1 Control Foxp3⁺ Regulatory T Cell Function Through Shared and Isoform-Specific Mechanisms

U. H. Beier et al.

Combined inhibition of distinct histone deacetylases enhances the suppressive effects of regulatory T cells.

PERSPECTIVE: How Growth Abnormalities Delay "Puberty" in *Drosophila*

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An insulin-like peptide delays metamorphosis of the fruit fly in response to injury or tissue overgrowth.

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20 June issue: <http://scim.ag/stm062012>

RESEARCH ARTICLE: DNazyme Targeting c-jun Suppresses Skin Cancer Growth

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FOCUS: Resurrecting DNazymes as Sequence-Specific Therapeutics

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Catalytic DNA molecules that target the transcription factor c-jun inhibit skin cancer growth in mice.

RESEARCH ARTICLE: The Structural Basis for Serotype-Specific Neutralization of Dengue Virus by a Human Antibody

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The mechanism of action of a serotype-specific natural human antibody against dengue virus has been identified.

RESEARCH ARTICLE: mTOR Inhibitors Synergize on Regression, Reversal of Gene Expression, and Autophagy in Hepatocellular Carcinoma

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RESEARCH ARTICLE: Recombinant MG53 Protein Modulates Therapeutic Cell Membrane Repair in Treatment of Muscular Dystrophy

N. Weisleder et al.

FOCUS: A Molecular Bandage for Diseased Muscle

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Recombinant human MG53 protein can increase membrane repair after injury in cells and can reduce pathology in animal models of muscle injury and muscular dystrophy.

SCIENCE CAREERS

www.sciencereers.org/career_magazine

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Can NIH Renovate the Biomedical Workforce?

M. Price

An NIH committee recommends overhauling training, increasing postdoc pay, and improving and expanding staff scientist positions.

<http://scim.ag/RenovateWorkforce>

Career Q&A: Equality for Quality

E. Pain

Curt Rice of the University of Tromsø discusses why helping women prepare for promotion is both right and smart.

http://scim.ag/QA_CurtRice

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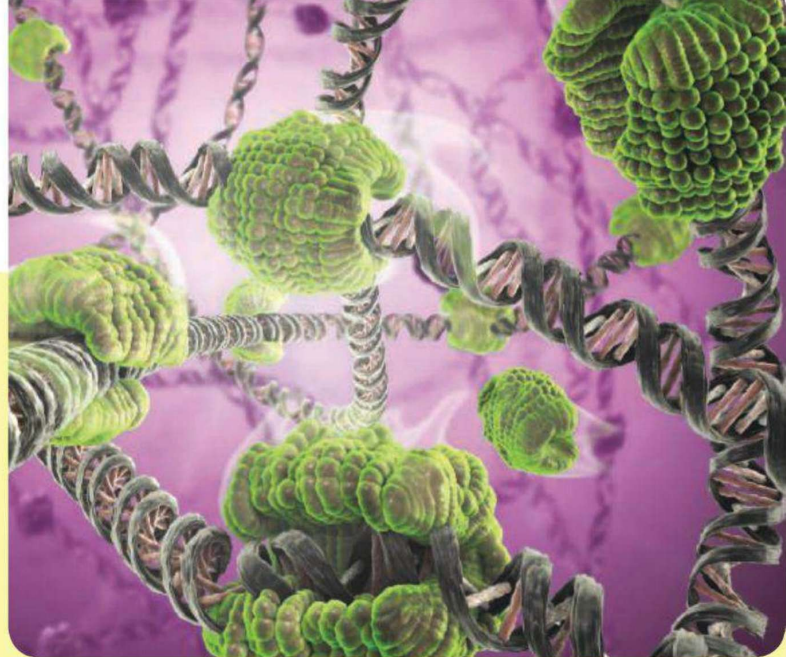
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Slide and Find >>

Transcription factors rapidly find their specific binding sites on chromosomal DNA. It has been proposed that searching is facilitated by complementing three-dimensional diffusion with one-dimensional diffusion along DNA. Such sliding on DNA has been observed *in vitro*, but whether and how far transcription factors slide along chromosomes *in vivo* is unclear. **Hammar *et al.*** (p. 1595) used single-molecule imaging to demonstrate that the *lac* repressor slides into its chromosomal operators in living cells. The average sliding distance was about 45 base pairs, and the repressor frequently slid over its operator before binding.



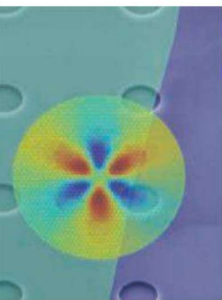
Master Regulator

Sympathetic neurons and the adrenal medulla are part of the autonomic nervous system, which is important in the control of a variety of bodily functions and in responses to stress. Interactions between the nervous system and the vascular system have been poorly explored. During development, sympathetic neurons and adrenal medulla cells are derived from the same precursors—neural crest cells—embryonic cohorts that undergo massive migration in the body. Using blood vessel-specific gene manipulation in chicken embryos, **Saito *et al.*** (p. 1578) revealed a role for the dorsal aorta in regulating the early migration of neural crest cells and later in development on the segregation of adrenal medulla and sympathetic neurons. The dorsal aorta expresses multiple soluble morphogenetic and growth factors that regulate complex morphogenesis in a spatiotemporal manner. Furthermore, in mice, the morphogenesis of the adrenal medulla was controlled both by the aorta and the adrenal cortex.

Straining Suspended Graphene

The electronic properties of graphene are best displayed by suspended sheets free from contact with an underlying substrate.

Klimov *et al.* (p. 1557) probed how deformation of suspended graphene sheets could lead to further tuning of its electronic properties with a scanning tunneling microscope; the graphene sheets could also be deformed via an electric field from an underlying electrode. Spectroscopic studies reveal that the



induced strain led to charge-carrier localization into spatially confined quantum dots, an effect consistent with the formation of strain-induced pseudomagnetic fields.

A Spike Inside the Dome

The transition temperature T_c of iron-based superconductors has a dome-shaped dependence on chemical doping, and the superconductivity that develops underneath may obscure a potential quantum critical point (QCP) residing at absolute zero. With the aim of detecting signatures of this quantum criticality, **Hashimoto *et al.*** (p. 1554; see the Perspective by **Sachdev**) measured the penetration depth of the pnictide series $\text{BaFe}_2(\text{As}_{1-x}\text{P}_x)_2$ as a function of x . A sharp peak right around the point where T_c has a maximum ($x = 0.30$) was observed, implying that the superfluid density diminishes sharply where one would expect it to be the most robust. This unusual finding is interpreted as a sign of a QCP at $x = 0.30$.

From Wnt Signals to Telomerase Activity

Telomerase activity is associated with stem cell renewal and cancers, whereas a decrease in telomerase activity is seen during cell differentiation and senescence. Wnt/ β -catenin signaling is also a critical regulator of stem cells, and deregulation of the pathway is associated with cancer. Now, **Hoffmeyer *et al.*** (p. 1549; see the Perspective by **Greider**) have found a link between these two pathways. In embryonic stem cells, β -catenin was able to regulate telomerase expression and activity directly. Similar observations were obtained in adult stem cells, a model of intestinal tumors, and human cancer cells.

Exploiting Defects in a Jam

Phase-change materials that can readily switch between crystalline and amorphous states are increasingly finding use in nonvolatile memory devices (see the Perspective by **Hewak and Gholipour**). Using high-resolution transmission electron microscopy, **Nam *et al.*** (p. 1561) show that for $\text{Ge}_2\text{Sb}_2\text{Te}_5$, the application of an electric field drives crystal dislocations in one direction, leading to their accumulation and eventual jamming, which causes the phase transition. **Loke *et al.*** (p. 1566) found that by applying a constant low voltage to $\text{Ge}_2\text{Sb}_2\text{Te}_5$, they could accelerate its phase-switching speeds, without harming the long-term stability of the switched state.

No More Fusion Confusion

Biophysical models explain membrane fusion as a sequence of steps—including membrane contact, formation of a fusion stalk (merger of proximal monolayers), development of contact between distal monolayers that may or may not expand (hemifusion), and, finally, rupture of this diaphragm resulting in the opening of a fusion pore. Biological membrane fusion reactions are often driven by so-called SNARE proteins. By using a reconstituted membrane fusion system, **Hernandez *et al.*** (p. 1581, published online 31 May) have now been able to correlate precisely the states of SNARE zippering with intermediate structures along the fusion pathway. The results suggest that a tightly docked state, with a membrane distance so close that no proteins fit in between them, represents a critical fusion intermediate as a consequence of SNARE zippering. This intermediate is incompatible with a SNARE-driven stalk or with a ringlike arrangement of SNAREs depicted in most current models of membrane fusion.

CREDITS (TOP TO BOTTOM): TREMANI/JOHAN ELF; NIKOLAI N. KLIMOV AND TENG LI, NIST/UMD

Cavity-Induced Minimum

Tuning the strength and range of interactions in cold atomic gases is crucial to their role as quantum simulators. Most atom-atom interactions are short-ranged. One way to extend the range is to couple the gas to an optical cavity, which can propagate interactions between atoms, making the interactions effectively long-ranged. This system has been used to observe a transition to a “supersolid” phase characterized by a checkerboard atomic density order. **Mottl et al.** (p. 1570, published online 17 May) used Bragg spectroscopy to measure the excitation spectrum of an ultracold gas of Rb-87 atoms as the interaction strength was varied. Consistent with theoretical predictions, a minimum was observed in the excitation energy, similar to that observed in roton excitations of the superfluid helium.

Tropical Carbon Loss

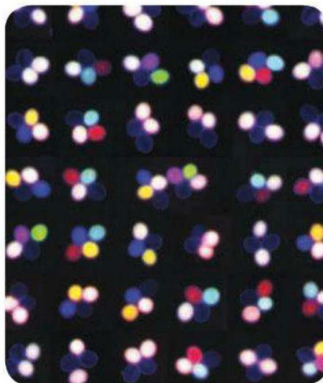
Accurate and precise measures of tropical deforestation and the resulting carbon emissions are needed in order to formulate climate policy. **Harris et al.** (p. 1573; see the Perspective by **Zarin**) used satellite observations of deforestation within the tropics of three continents to estimate that gross annual carbon emissions were approximately 0.8 Pg C yr^{-2} ($\text{Pg} = 10^{15} \text{ g}$) for the years 2000 to 2005, from the loss of 43 million hectares of forest. This result, which is about one-third of some previous estimates, should serve as a baseline for future assessments of changes in the rate of loss of tropical forests.

Plants That Eat Animals

Apart from some spectacular exceptions, such as pitcher plants and Venus fly traps, most plants are thought to acquire nitrogen passively from microbial decomposition and the activities of nitrogen-fixing bacteria. *Metarhizium* species are common endophytes—fungi that live within plant tissues without causing disease. This genus is also found ubiquitously in soil, where they parasitize insects. In a series of microcosm experiments, **Behie et al.** (p. 1576) investigated whether these fungi could couple their endophytic life-styles with their parasitic modes and be a conduit by which plants could obtain nitrogen from animals. Radio-labeled moth larvae were added to the microcosms in which bean and grass plants were grown, and when the larvae were inoculated with fungi, it was only a matter of days before the nitrogen label was detected in the plants.

No Crossing Over

To ensure the correct division of chromosome during the reduction division of meiosis, homologous chromosomes undergo double-strand breaks that—through crossing over and recombination—link the homologs together (and importantly introduce diversity into the genomes of gametes). But only a minority of these crossovers results in recombination—most are directed into non-crossover pathways. **Lorenz et al.** (p. 1585), working in the yeast *Schizosaccharomyces pombe*, and **Crismani et al.** (p. 1588), working in the higher plant *Arabidopsis thaliana*, looked for the factors that limit crossovers and promote non-crossover pathways. The homolog of the human *Fanconi anemia complementation group M (FANCM)* helicase protein was found to be a major meiotic anti-recombinase, which could drive meiotic recombination intermediates into the non-crossover pathway.



Blasting Through

The fungus that causes rice blast disease, *Magnaporthe oryzae*, can lead to devastating reductions in rice yields. *M. oryzae* enters the plant by developing specialized infection structures called appressoria. Appressoria generate enormous internal turgor pressure that somehow creates invasive forces that physically break the plant cuticle. **Dagdass et al.** (p. 1590) found that a toroidal (doughnut-shaped) filamentous actin network forms at the base of the appressorium at the precise point where the penetration peg, which ruptures the rice leaf cuticle, will emerge. This network is scaffolded by means of four septin guanosine triphosphatases, which form a dynamic ring structure that colocalizes with F-actin. The findings reveal how fungi translate extreme pressure into localized physical force.

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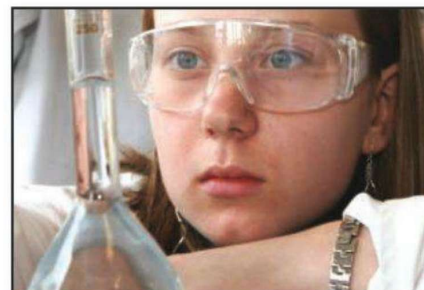
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Universities, Key to Prosperity

BY ALMOST ANY MEASURE, U.S. RESEARCH UNIVERSITIES ARE CONSISTENTLY RANKED AMONG THE BEST in the world. Beginning with the Morrill Act (Land Grant College Act) of 1862, federal and state policies have underscored the nation's commitment to building the finest research institutions. As such, U.S. universities have been the incubators of the nation's prosperity. E. I. du Pont de Nemours and Company, which I once headed, is a great example. One of its key products, nylon, was made possible because of a Harvard-educated polymer researcher who brought his knowledge to DuPont. Today, that kind of connection is everywhere, from high-tech Silicon Valley startups to cutting-edge East Coast biomedical facilities. The talent and knowledge produced by research universities underpin many of the finest U.S. achievements, from seeding the modern agricultural system to enabling the World Wide Web. But new challenges are putting these important national assets in danger. The National Research Council (NRC) committee that I chaired to assess the capacities of U.S. universities has now released recommendations for maintaining their role in national prosperity.*

As the United States struggles to recover from a prolonged financial crisis, government at every level has been forced to make difficult choices. Public funding for research has been flattened or cut while other nations invest more in research. U.S. universities have had to raise tuition, threatening to put college education out of reach for many. But our committee concluded that, especially in these tough times, the United States cannot afford to defer investment in research universities. If the nation is willing to renew its commitment to keeping these institutions the best in the world, they will lead the way to the next generation of scientific and technological breakthroughs that propel U.S. prosperity, just as they have in the past. It is critical to revitalize the partnership between our research universities, government, and industry.

The NRC report recommends 10 actions that the nation must take to shore up its research institutions, and all partners must play their part. For the federal government, this means providing sustainable funding for university research and graduate education within the context of increasing national R&D funding from all sources—government, private-sector, and philanthropic—to 3% of the nation's gross domestic product. While sustaining national investment in biomedical research and other key areas, Congress should increase support for research already authorized by the America COMPETES Act, thereby doubling the funds for basic research. States and businesses must also renew commitments to their home institutions, which are critical to their competitiveness in an innovation-based economy. State appropriations per enrolled student have declined by 25% or more over the past two decades. As the economy recovers, states should restore appropriations to the levels that maintain high-quality institutions. U.S. businesses, which depend on universities for technology and talent, must also provide long-term financial support and regionally build strong, enduring partnerships. The federal government should provide tax incentives for businesses to partner with universities early in the research process, and businesses should use research results to create U.S. jobs.

For research universities, we recommend that, along with government effort to reduce unnecessary regulations, investments should be made in infrastructure—particularly cyber-infrastructure—to improve productivity in administration, research, and academic programs. And to continue to attract the best students, universities must reduce attrition rates in science and engineering majors and shorten the time it takes for graduates to obtain degrees.

Such sweeping changes aren't going to happen overnight. But our children and grandchildren deserve the same opportunities that we had. History tells us that maintaining a commitment to our universities over the long haul, through good times and bad, is the best way to ensure that they will.

— Charles O. Holliday

10.1126/science.1225457

**Research Universities and the Future of America: Ten Breakthrough Actions Vital to Our Nation's Prosperity and Security* (National Academies Press, Washington, DC, 2012); available at www.nap.edu/catalog.php?record_id=13396.



Charles O. Holliday is the former chairman and chief executive officer of E. I. du Pont de Nemours and Company and is chair of the committee that wrote the NRC report.





EDUCATION

Respect My Authority

Graduate teaching assistants (TAs) are regular fixtures in science classrooms; however, their status and authority as teachers remain ambiguous. Kendall and Schussler present a thorough comparison of TAs and professors from the perspective of undergraduates in order to enhance TA professional development. Online surveys designed to evaluate skills such as leadership, personalization, involvement, and task orientation were sent to students enrolled in major and nonmajor biology courses that had a laboratory component taught by TAs, with lectures taught by faculty. Weakness in one instructor type appeared to be balanced by the strengths of the other: Although professors were perceived as confident, organized, boring, and out of touch, TAs were perceived as uncertain, hesitant, approachable, and able to personalize teaching. Additionally, although undergraduates perceived professors as having more knowledge of the curriculum, they favored the instructional style of TAs. Professional development programs can capitalize on these findings by increasing opportunities for TAs and professors to teach collaboratively, which should help both parties address perceived weaknesses. — MM

CBE—Life Sci. Educ. **11**, 187 (2012).

CHEMISTRY

Phenol Coming and Going

There are, broadly speaking, two ways to drive electric current. One is to separate the components of a chemical reaction so that the electrons travel separately from the ions; this is the working principle of a battery and yields direct current (dc). The other method is to rotate a wire loop through a magnetic field, which generates alternating current (ac). For the most part, when the process is inverted to drive chemistry via electricity, a dc approach is the most straightforward to implement, because it essentially runs a battery in reverse. However, ac-mediated chemistry can sometimes prove advantageous, as Lee *et al.* demonstrate in an

exploratory small-scale electrolytic synthesis of phenol from benzene. The authors set up their cell so that benzene oxidation occurred at both the anode and the cathode, propelled in the former case by preliminary water oxidation and in the latter by oxygen reduction. Coupling vanadium oxide electrode catalysts with an indium-doped tin diphosphate solid electrolyte, they examined each half reaction in turn and found evidence for distinct mechanisms based on differences in temperature and potential dependences and Raman spectroscopic data. An alternating frequency of 30 Hz afforded optimal selectivity for the phenol product overall. — JSY

Angew. Chem. Int. Ed. **51**, 10.1002/anie.201202159 (2012).

BIOMEDICINE

The Healing Membrane

The omentum is a fold of smooth membrane that forms part of the lining of the abdominal cavity, extending from the stomach to adjacent lower abdominal organs. Although omentum has been observed to promote tissue healing and revascularization and to control inflammation when surgically grafted to injured sites, its mechanisms of action are not well known. Shah *et al.* found that in a mouse model of lung injury, animals injected with cells prepared from “activated” omentum showed a decrease in tissue inflammation, T cells, and proinflammatory cytokines in the damaged lung. Cells prepared from activated omentum tissue included mononuclear myeloid suppressor cells that could block the proliferation of effector/regulatory T cells, including T_H17 cells. Furthermore, activated omentum contained cells that could differentiate into various cell types under specific tissue culture conditions, including epithelial cells and osteoblasts, suggesting that the membrane harbors mesenchymal stem cells. How the omentum recruits these different cell types is not clear, but their presence in the membrane may have implications for omentum’s clinical use in tissue repair and healing. — LDC

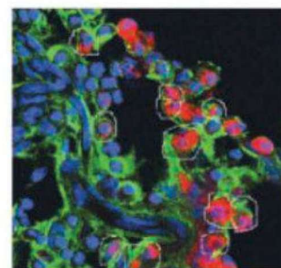
PLOS One. **7**, e38368 (2012).

CELL BIOLOGY

The Matter of the Heart

Cell proliferation and differentiation are directed by specific transcription factors and signaling molecules, but recent studies show that matrix stiffness can also have a major effect. Kshitiz *et al.* used poly-

acrylamide gels with different rigidity values to show that substrate stiffness is important for the morphogenesis and differentiation of human and rat cardiosphere-derived progenitor cells (CDCs) toward the endothelial fate. The optimal rigidity matched that of heart tissue. When compared to cells cultured on glass, cells that had been cultured



Continued on page 1484

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things you didn't
(and 3 you probably
shouldn't) know
about some of
your most
respected
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on a myocardium rigidity-mimicking substratum (MRS) showed better survival and better integration into blood vessels, and expressed factors associated with endothelial differentiation when applied to a rat model of myocardial infarction. A c-Kit-positive cell population was implicated as the source of CDC-derived endothelium, with p190RhoGAP involved in this process via RhoA-dependent and independent mechanisms. Down-regulation of p190RhoGAP was sufficient to bias cell differentiation toward the endothelial lineage, whereas its up-regulation favored the cardiomyogenic lineage. Thus, the rigidity of the cell environment in cardiac tissue affects cell proliferation and differentiation, which could be important when considering therapeutic interventions. — BAP

Sci. Signal. **5**, ra41 (2012).

MICROBIOLOGY

Living with *Legionella*

Legionnaire's disease is an adventitious severe infection of humans caused by a ubiquitous bacterium that naturally lives within aquatic protozoa that colonize inadequately maintained water cooling systems. *Legionella pneumophila* uses the IVB Dot/Icm translocation system to introduce proteins into the host cell that remodel an intracellular vacuole to make it habitable. The bacterium then replicates within the modified host cell vacuole. The Dot/Icm system has a large repertoire of translocatable substrates, which appears to endow this pathogen with a wide host range, allowing it even to colonize mammalian macrophages. In a series of evolution experiments, Ensminger *et al.* discovered that when grown exclusively in mouse macrophages for several months, *L. pneumophila* improved its ability to replicate in this abnormal host cell to the extent that it failed to thrive when returned to amoebae. Population genotyping revealed mutations in the flagellar regulator and defects in lysine biosynthesis that could act to increase uptake into and replication within macrophages. — CA

PLoS Pathogens **8**, e1002731 (2012).

MATERIALS SCIENCE

Refusing to Order

In magnetic materials, neighboring electron spins are aligned and point in either the same or opposite directions; the latter order is called antiferromagnetic. If the spins are arranged in, e.g., a triangular lattice, the antiferromagnetic condition cannot be satisfied everywhere simultaneously, and it is in principle possible for the system to remain disordered down to

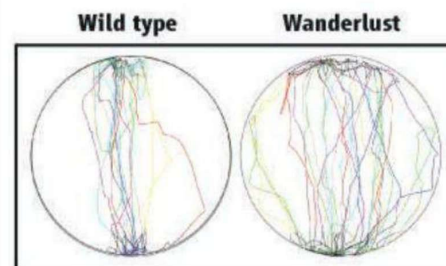
the lowest temperatures, giving rise to exotic ground states. However, other types of order or distortions usually intervene. Shekelton *et al.* engineered the material $\text{LiZn}_2\text{Mo}_3\text{O}_8$, where clusters of Mo_3O_{13} are arranged in planes of triangular lattices. These clusters have an electron delocalized across the three Mo atoms, which makes the electronic structure stable against symmetry-breaking distortions; the clusters have an effective spin 1/2. Neutron diffraction and heat capacity measurements indicated that the material does not order magnetically down to 0.1 K and that at 96 K, two-thirds of the effective magnetic moments form singlets. Further experiments are needed to elucidate the nature of the ground state, but existing data suggest that the singlets are dynamic, forming an exotic condensed valence bond state, similar to the resonating valence bond state proposed to describe the high-temperature superconductivity of cuprates. — JS

Nat. Mater. **11**, 493 (2012).

NEUROSCIENCE

Restless Flies, Fragmented Sleep

Restless leg syndrome (RLS), characterized in humans by an irrepressible urge to move the legs, has been linked to the gene *BTBD9* and its cognate protein. The BTBD9 protein is



widely distributed through the central nervous system, where it seems to function as an adaptor for ubiquitin ligase. Freeman *et al.* have now identified a similar gene in *Drosophila* and analyzed its function. Deletion of the gene in *Drosophila* resulted in peripatetic flies with shortened life spans. These mutant flies also showed disrupted sleep patterns, with normal totals of sleep time delivered in fragmented, shortened bouts. Time spent walking, on the other hand, was greater than normal. Targeted knockdown experiments revealed a role for dopaminergic neuron subtypes. Treatment with a dopamine agonist reduced the flies' symptoms. In tissue culture cells, BTBD9 affected iron mobilization pathways through its interaction with iron regulatory protein-2. — PJH

Curr. Biol. **22**, 10.1016/j.cub.2012.04.027 (2012).

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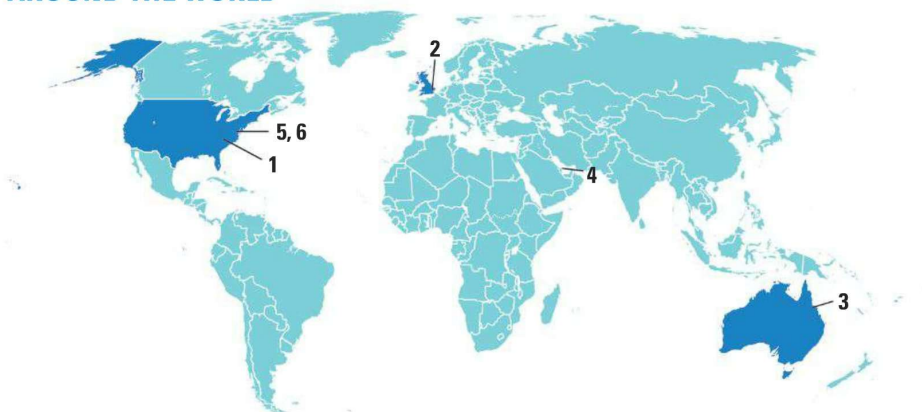
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AROUND THE WORLD



Raleigh, North Carolina 1

Legislating Sea Level Rise

By a 34-to-11 vote, on 12 June North Carolina's Senate approved a controversial bill, HB 819, which requires North Carolina's Coastal Resources Commission to base predictions of future sea level rise along the state's coast on linear rates of increase rather than allowing for accelerating events, such as the melting of polar ice sheets. The bill's supporters say that predicted acceleration would

be expensive to plan for and is based on flimsy data.

Climate scientist Robert Jackson of Duke University in Durham, North Carolina, who spoke before the Senate committee that approved the bill on 7 June, says the legislation would force

state planners to downplay the potential impacts of global climate change. Models using linear rates of sea level increase based on past changes forecast 0.2 meters of sea level rise along the North Carolina coast by 2100, he says; in contrast, models that allow for accelerated rates forecast a 1-meter rise.

The bill still needs to be approved by the North Carolina House of Representatives and signed by the governor to become law. <http://scim.ag/NCsealevel>

London 2

U.K. Panel Backs Open Access

A U.K. government report says open-access journals should be "the main vehicle for the publication of research" but

warns they will cost the country more than \$80 million per year.

Most scientific papers are published by journals that charge a subscription fee. Publishers such as the Public Library of Science (PLOS) and Biomed Central have pioneered an alternate model in which authors pay a fee for publication and the paper is then freely available online.

The report, published on 19 June, suggests that journals are increasingly adopting the PLOS model. But in the interim, the report cautions, universities may end up paying author fees for some journals on top of subscription costs for others. Timothy Gowers, a mathematician at the University of Cambridge and an open-access advocate, criticizes the report for being "more concerned with the openness issue than with making a serious attempt to reduce costs."

<http://scim.ag/UKopenacc>

Australia 3

Australia Creates World's Largest Network of Marine Reserves

At Rio+20 this week, Australian Prime Minister Julia Gillard can hold her head high: Last week, her government unveiled the final plan for the world's largest network of marine parks. After 10 years of planning, the idea needs to pass one more 60-day comment period before coming into force at the end of the year. Protected areas will increase more than four-fold, to 3.1 million square kilometers.

"This sets a new benchmark," says Terry Hughes, director of the Australian Research Council Center of Excellence for Coral Reef Studies at James Cook University in Townsville. Critics of the plan include the fishing lobby, which argues that parks place undue pressure on unprotected fisheries and do not

increase stocks, an argument rebuffed by a recent paper in *Current Biology* that showed marine reserves restock fisheries up to 30 kilometers away.

The park increases the number of Australia's marine reserves from 27 to 40 and covers a wide range of habitats. Only one-third of the area will be fully excluded from fishing or gas and oil exploration, protecting habitats and species from humpback whales to green sea turtles; the other two-thirds will be managed to allow varying degrees of use.

Manama 4

Bahrain Sentences Medics

Bahrain's highest court released its final verdict on 14 June in the case of 20 medics accused of fomenting revolution in the Persian Gulf state. While most of the charges were dropped—the prosecution relied on confessions that the medics claim were extracted through torture—the defendants still face jail time ranging up to 5 years.

The verdict is a small victory for the International Human Rights Network of Academies and Scholarly Societies. The consortium of scientists, doctors, and engineers has been pressuring Bahrain's king to release the medics and clear them of all charges.

Meanwhile, Bahraini doctors say that they are still in danger of political reprisals. "Patients should have access to medical care without the fear of arrest, and the doctors should be able to treat any patient without the fear of prosecution," says Amal Habib, an ophthalmologist who has fled Bahrain and is seeking asylum abroad.

<http://scim.ag/Bahraindocs>

Washington, D.C. 5

Senate Panel Gives NIH \$100 Million Boost for 2013

The Senate Appropriations Committee last week approved a modest \$100 million raise for the National Institutes of Health (NIH) in 2013. The 0.3% bump, to \$30.723 billion, is better than the president's request for no increase but disappointing to the research community.

The bill provides \$40 million for the Cures Acceleration Network, four times its current budget (but \$10 million below the president's request). It disregards the president's proposal to cut \$50 million



from the Institutional Development Award (IDeA) program, which boosts competition for grants in poorer states, but makes a requested \$28 million trim, to \$165 million, for the National Children's Study.

"We're appreciative of any increase," says Jennifer Zeitzer, director of legislative relations for the Federation of American Societies for Experimental Biology. Still, she says, "it's going to be difficult for people in the research community."

The bill must still be approved by the full Senate. The House appropriations panel was expected to introduce its version of the bill as soon as this week. <http://scim.ag/NIHboost>

Washington, D.C. 6

NRC Committee Finds That Humans Are Triggering Quakes

Fracking for natural gas and oil, formally known as hydraulic fracturing, has been responsible for only one or two earthquake-triggering episodes, a committee of the National Research Council (NRC) concluded in a report released on 15 June. But hundreds of felt quakes in 13 states have been produced by the deep injection of wastewater from fracking and other industrial activities, as well as the creation of geothermal energy sites, the enhanced recovery of oil and gas, and even the simple extraction of oil and gas. And pumping carbon dioxide captured from power plants into the ground "may have potential for inducing larger seismic events" because of the large volumes involved, the committee found.

Assessments of the risk of induced seismicity "should be undertaken before operations begin in areas with a known history of felt seismicity," the committee wrote. Such preinjection studies have generally not been done in the past, although some new state regulations are moving in that direction. <http://scim.ag/NRCfrack>

NOTED

>The United States is back at the top of the supercomputing list. IBM's Sequoia, at Lawrence Livermore National Laboratory in Livermore, California, beat out Japan's Fujitsu to regain the title of **the world's most powerful computing system, as ranked by the industry**-standard Top500 list, released 18 June at the International Supercomputing Conference in Hamburg, Germany.



Fewer Foxes, More Lyme Disease?

The increase in Lyme disease over the past 30 years in the United States has often been blamed on resurging populations of deer because they are a major host for adult ticks carrying the disease-causing bacteria. But a study published online this week in the *Proceedings of the National Academy of Sciences* puts the blame instead on the decline of red foxes, which are being displaced by coyotes. Researchers led by ecologist Taal Levi of the University of California, Santa Cruz, examined the incidence of Lyme disease in Pennsylvania, Virginia, Wisconsin, Minnesota, and New York, and found that a higher incidence of Lyme disease correlated with the scarcity of red foxes. Foxes are very effective at catching mice and other rodents that infect young ticks with Lyme-causing bacteria. "The strength of the data is impossible to ignore," comments ecologist Richard Ostfeld of the Cary Institute of Ecosystem Studies in Millbrook, New York.

NEWSMAKERS Three Q's



Boyden

At his synthetic neurobiology lab at the Massachusetts Institute of Technology, **Edward Boyden** is exploring the circuitry of the brain. On 19 June, Boyden received the inaugural A. F. Harvey

Engineering Research Prize of £300,000 (\$470,000), awarded by the Institute of Engineering and Technology, an international professional society, for his work in the development of optogenetics, a powerful technique that enables precise control of neuronal activity with laser light.

Q: What are the latest developments in your lab?

A: In collaboration with mechanical engineers at [the Georgia Institute of Technology], we've built robots that automate recordings from inside nerve cells, which can be scaled up to record from hundreds or thousands of cells. We're also developing the ability to characterize neural network computations by simultaneously controlling and reading out brain activity.

Q: What's the future of optogenetics?

A: I'm interested in devices that can record from, and deliver information to, multiple points in the brain, with a computer that processes the information and determines exactly what needs to be restored [to correct a brain disorder]. Devices that interface with entire neural circuits at single-cell resolution would allow understanding of how the cells work together ... and how they go awry in diseases.

Q: What are the biggest challenges?

A: The most obvious challenge is how to deliver genes encoding light-sensitive proteins to specific cells in the human brain. Gene therapy trials are promising, but we need to understand its consequences fully. Another question is [whether] these genes (obtained from microbes) will elicit immune responses when introduced into the human body.

'Protein Detective' Wins Körber Prize

Matthias Mann, 52, a physicist and bioinformatician at the Max Planck Institute of Biochemistry in Martinsreid, Germany, has won the 2012 Körber European >>

>>NEWSMAKERS

Science Prize for his groundbreaking work on the proteome, the entire complement of proteins in a living organism. The prize comes with an award of €750,000.



Mann

As a Ph.D. student at Yale University, Mann worked with analytical chemist John Fenn, who received the Nobel Prize in chemistry in 2002 for his work on developing electrospray ionization, which makes it possible to

measure proteins using mass spectroscopy methods, once the province primarily of chemists and physicists.

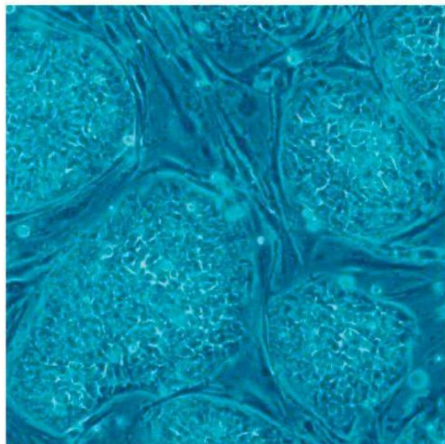
Mann refined the technique so that all the proteins in a cell could be analyzed at once; in 2010, he and his team were the first to successfully sequence the complete proteome of a living organism.

"Matthias is a splendid choice for this high award," says geneticist Gilbert Omenn, of the University of Michigan, Ann Arbor. "He is a remarkable innovator ... and he and his laboratory have made numerous important biological applications."

FINDINGS

Stem Cell Hope for Vision, Brain

Bone marrow transplants have been used for decades, but research presented last week in Yokohama, Japan, at the annual meeting of the International Society for Stem Cell Research (ISSCR) confirmed that scientists



are making progress at developing more innovative stem cell therapies.

A research team from the RIKEN Center for Developmental Biology in Kobe pre-

BY THE NUMBERS

287 million tonnes The collective weight of the world's adult human population, according to a *BMC Public Health* study. Fifteen million tonnes of that is due to overweight people.

\$100 billion Amount U.S. research universities need in new federal support over the next decade to remain the best in the world, according to a report released 14 June by the National Academies.

£20,000 Cash prize offered with the newly announced Wellcome Trust Screenwriting Prize, intended to help develop films inspired by medicine and biology.

sented animal studies indicating that stem cells created from a person's own cells could be turned into retinal cells that treat a form of age-related macular degeneration. And StemCells Inc. of Newark, California, reported encouraging results from transplants of human neural stems into four infants with Pelizaeus-Merzbacher disease (PMD), a progressive and fatal disorder in which a genetic mutation inhibits the normal growth of myelin, a protective material that envelopes nerve fibers in the brain.

In the clinical trial conducted by researchers at the University of California, San Francisco, magnetic resonance imaging taken 18 months after the transplants indicated the formation of new myelin around axons, and clinical observations of treated infants indicated that their motor functions remained stable or enjoyed modest gains. The company is planning larger trials; an official says that if the therapy proves efficacious, it could lead to treatments for multiple sclerosis, cerebral palsy, and Alzheimer's disease. <http://scim.ag/primestem>

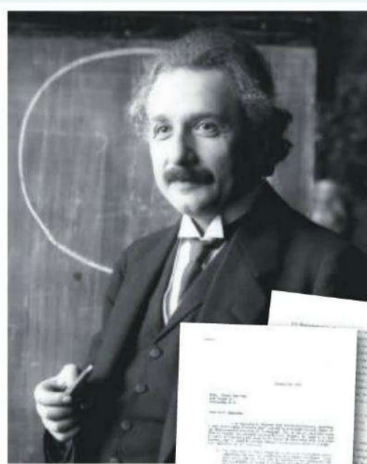
Letter Details Einstein's Post-War Passion

A cleanup of the archives of the Jewish Telegraphic Agency in January yielded an unexpected treasure for the 95-year-old news service: hand-typed correspondence between JTA's founder, Jacob Landau, and Albert Einstein. Among the yellowing letters was a 20 January 1947 statement from Einstein on scientists' role in military research—a hot topic in the wake of World War II and the wartime use of atomic weapons.

"Non-cooperation in military matters should be a vital part of the moral code of basic scientists," Einstein wrote, adding that keeping basic discoveries secret "would seriously harm science."

Einstein had expressed similar antiwar views prior to writing that letter, but it does shed new light on the physicist's views "on the relationship of science and state," says Harvard University historian Peter Galison. Einstein writes, for example, that for science, "moral law is above any obligation to the state."

The letters also illuminate Einstein's ties to Landau, who founded the news service to cover the Jewish diaspora in 1917. Einstein's long-standing friendship with the pressman—who named his son Albert E. Landau—helped shape the scientist's views on the Holocaust, Israel, and other topics. To raise funds for a new Web site, JTA auctioned the letters at Sotheby's last week, where they fetched \$34,375.



Science LIVE

Join us Thursday, 28 June, at 3 p.m. EDT for a live chat on **whether we're training too many graduate students.** <http://scim.ag/science-live>

BIOMEDICAL CAREERS

Young Researchers Deserve More Support, Reviews Say

Two new reports say that the U.S. government needs to change how it supports doctoral training so that students can receive better mentoring and research universities can produce the right mix of skilled workers the nation needs. The problem, these reviews say, is that the U.S. system is churning out candidates for academic jobs that don't exist.

The need to rebalance current federal support was highlighted in two unrelated documents released last week. The first is a draft report from an advisory committee to the National Institutes of Health (NIH), chaired by Princeton University President Shirley Tilghman, which was asked for recommendations on how to train and retain the next generation of biomedical scientists. The second came from a National Academies committee chaired by former DuPont CEO Charles Holliday in response to a request from Congress for recommendations on how to maintain the excellence of U.S. research universities (see p. 1491).

Although the two groups worked independently and the timing of their reports was coincidental, both reached the same conclusion: Supporting graduate students from the research grants held by their advisers—the current dominant mode of federal support—makes them too dependent on their mentors. The reports recommend beefing up two other mechanisms—competitive fellowships and institutional traineeships—designed to give students more independence and flexibility to develop into productive scientists, and more opportunities to learn about careers outside academia.

At the same time, both reports emphasize that rebalancing the training system should not jeopardize the productivity of principal investigators, who rely on students to conduct most of the experiments in their labs. Studies conducted by NIH show “that students who have been supported on training grants have better career outcomes than control groups of students who are not supported on training grants,” Tilghman said. “If there is a finite pot of money that is being used to support training of graduate students, then NIH is far better off having those dollars invested in peer-reviewed training.”

The NIH report that came out last week is the second one in which Tilghman has sounded a similar alarm about graduate training. If anything, she said, the imbalance has gotten worse since she chaired a 1998 National Academies panel that produced the report, *Trends in the Early Careers of Life Scientists*. It came out just as NIH was beginning

ber of new academic positions.

“What concerns me is that, under the current system, you are developing people who can't move away from the research of their major professor when they become independent investigators,” says Enriqueta Bond, a member of the National Academies panel and former head of the Burroughs Wellcome Fund, a strong supporter of graduate fellowships and traineeships in the life sciences. It's better to rely on well-run training programs, she says, because these can be “crafted to do much more than just purchase a unit of research.”

To correct the imbalance in the current system, the Tilghman report recommends that



Shirley Tilghman

NIH Advisers' Recommendations

- Graduate students limited to 6 years of NIH funding
- Training to include management and entrepreneurship
- Increase support for training grants and fellowships, not the number of positions supported
- Increase postdoc stipends from \$39,264 to \$42,000
- NIH-funded institutions must track student careers
- Move scientists into independent careers faster
- Encourage universities to support staff scientists
- Harmonize the jumble of NIH training programs

a 5-year doubling of its budget. The increased funding in that period caused the number of Ph.D. students supported by research grants to soar while the number of students on traineeships and fellowships remained relatively static (see graph).

By providing their own source of funding, fellowships put students in the driver's seat. They are portable, meaning that the student can switch labs without losing financial backing. Traineeships, although they are awarded by the institution, are the result of a national competition that requires a graduate program to meet high standards for the type of mentoring it will provide. That could include career guidance for jobs outside of academia.

In contrast, students funded through research assistantships run the risk of being treated as a source of labor rather than as scientists in training. In addition, Tilghman said, students who follow narrowly in the footsteps of their advisers typically learn only one skill set: the academic one. That's a severe disadvantage in a world where the supply of new Ph.D. students each year far exceeds the num-

ber of new academic positions. “We are, I think, unanimously of the view that the most effective training dollars NIH has to extend are those in its training grants,” Tilghman said. “We think this will increase the overall quality of training of graduate students around the country.”

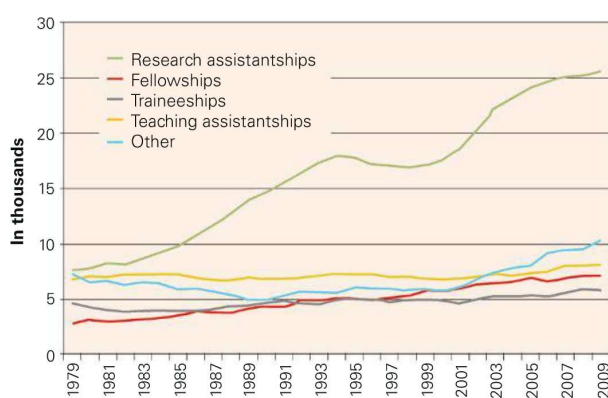
Some scientists worry that such a shift could make their labs less competitive in the never-ending hunt for funding. “One wants to be sure that the principal investigators who are supposed to be doing the research continue to have enough flexibility to be able to support the research they want to do,” said Robert Horvitz, a biologist at the Massachusetts Institute of Technology in Cambridge and a member of the advisory committee to the NIH director, which commissioned the study from Tilghman's working group. Taking away that flexibility, he argued, could reduce research productivity.

Judith Bond, incoming president of the Federation of American Societies for Experimental Biology, thinks that upping the number

of traineeships so that NIH can peer-review students' mentoring would overstep the government's role in graduate training. "Oversight of student training should be left to the universities, not the federal government," said Bond, a biochemist at Pennsylvania State University, Hershey.

Following the presentation, NIH Director Francis Collins told the committee, "We do need to do something—that seems quite clear." He suggested that some pilot-scale experimentation would be prudent to assess the consequences of putting more resources into training grants. "It would be a very good thing before we do something that's more systematically disruptive in ways that we didn't intend," he said.

The National Academies report, which



Explosive growth. During the boom years, the number of basic research assistantships attached to NIH grants increased rapidly.

focused on all disciplines and looked at many other issues besides doctoral training, suggests a politically more palatable solution than cutting the number of students on research grants. It would increase the total pool of federal support, with the new money going for

students on traineeships and fellowships. Specifically, it recommends establishing 5000 new fellowships and traineeships, at a cost of \$1.6 billion a year. The increase, it says, would reverse a recent decline in the total number of graduate students on federal support and restore the number to its 2006 peak of 84,000.

Noting that some senior scientists are resisting the Tilghman panel's recommendations, Enriqueta Bond says that she's not surprised: "I'd expect that pushback," she says. "After all, the students are doing their research."

Tilghman vows to forge ahead. "The only time when it's going to be possible to make hard decisions that would actually have a long-term, beneficial effect on all the players in the biomedical workforce is actually during tough times," she said. "Doing nothing, in my view, is not an option."

—MICHAEL PRICE

Michael Price is a writer with *Science Careers*. With reporting by Jeffrey Mervis.

EQUAL OPPORTUNITY

Action Urged to Curb Racial Bias in NIH Grants

To counter a racial tilt in the funding of basic research grants, the National Institutes of Health should launch a "continuous" review and take remedial steps against bias, a working group told NIH Director Francis Collins at a meeting on 14 June. These recommendations, laid out in a draft report to the NIH Advisory Committee to the Director, are aimed at correcting what the working group calls a "disturbing discrepancy": Black applicants are less likely to win independent investigator grants (R01 awards) than whites.

The panel—co-chaired by Reed Tuckson, executive vice president and chief of medical affairs at UnitedHealth Group in Minnetonka, Minnesota, and John Ruffin, director of NIH's Institute on Minority Health and Health Disparities—laid out a wide-ranging agenda. But it offered few specifics and no cost estimates. Tuckson told reporters that it's too soon to nail down such details. But he said he would like to see NIH and other federal agencies get together on a major, multiyear effort to support institutions that train minority scientists. Collins asked his advisory group to dig into the issue after a study by economist Donna Ginther of the University of Kansas,

Lawrence, and colleagues found that black applicants for NIH grants in 2000 through 2006 were significantly less likely (by 10 percentage points) to be funded than white applicants (*Science*, 19 August 2011, p. 1015). Blacks were also less likely to apply for basic science funding, Ginther found. When the

The report notes, however, that "analyses and discussions did not point to a single, definitive cause for NIH funding disparities."

Still, the panel says, NIH needs to gather data and take action. It should create a new position of chief diversity officer, with "a suitable budget." It should set up a new working group of behavioral and social scientists to do research—for example, to examine reviewers' written comments on applications. NIH should also conduct pilot testing of "bias/diversity awareness training" for NIH review staff. And the agency should experiment with anonymous reviews, blanking out applicants' identities and institutions. Most importantly, NIH should establish mentorship networks to assist minority students early in their school careers and help fund more undergraduate scholarships.

Collins said during the advisory committee meeting that he is committed to doing whatever it takes to reduce disparities in grant success rates. "This is a very serious issue," Collins said. "To have this circumstance continue ... is simply unacceptable."

—MICHAEL PRICE

Michael Price is a writer with *Science Careers*.



Fair science. Reed Tuckson (left) and John Ruffin, working group co-chairs, argue that a bold NIH initiative is needed to ensure racial parity in grant funding.

working group examined a set of grants from 2006 to 2010, it discovered similar biases.

Of "particular significance," the working group states, is that 73% of the applications from black researchers in 2006 through 2010 were rejected without a full discussion—or in NIH jargon, "triaged"—while 59% of applications from whites were rejected this way.

Making the case. William Frist argues for “growing the pie” as panel chair Charles Holliday (*far right*) looks on.



U.S. RESEARCH UNIVERSITIES

Panel Says More Money, Fewer Rules Are Best Ways to Stay Ahead of Pack

The U.S. higher education establishment couldn't be happier with a new report that repeats what it has said for years: Research universities are powerful engines of innovation for the U.S. economy. But it may be extremely hard for fans of those schools to persuade state and national legislators to approve the additional fuel that the report says is needed to keep that engine running smoothly.

In June 2009, four members of Congress who are active on national science policy issues asked the National Academies to come up with a top 10 list of the steps needed to “maintain excellence in research education.” Last week, a blue-ribbon panel of business leaders, university presidents, and prominent scientists convened by the academies delivered its answer: Give those elite institutions more money, with fewer strings attached, and they'll find ways to be more efficient in training students and operating their campuses.

That prescription has drawn a predictably enthusiastic response from higher education officials and groups. “It's the right report at the right time,” says Hunter Rawlings of the Association of American Universities. “Public research universities are at risk, and the nation must move to protect these vital assets,” chimes in Peter McPherson of the Association of Public and Land-Grant Universities.

Those who requested the report are more muted in their reaction. The chair of the House of Representatives science com-

mittee, Representative Ralph Hall (R-TX), notes that “more federal support ... will be difficult to achieve in these challenging economic times.” But Hall applauds the report's call for “other ways to strengthen research universities, including reducing bureaucratic and regulatory requirements.” Senator Lamar Alexander (R-TN), who spoke with the committee during its deliberations, said simply that he's “looking forward to reviewing the report's findings.” An aide to Senator Barbara Mikulski (D-MD) said her boss would have no comment at this time.

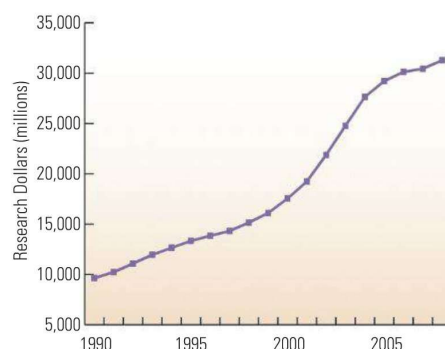
The report itself isn't shy about what the government can do for the country's 200 or so research universities. It proposes a new, \$70 billion “strategic investment” over 10 years, including \$2 billion a year for 2000 new faculty chairs for young researchers and

the rest for expanded computing capabilities. It calls for a \$1.6-billion-a-year boost in support of graduate training (see p. 1489), as well as the cash to meet a promised 10-year budget doubling for three key federal research agencies. And it suggests that states return to 1990s levels of per-student support for higher education after several years of declining funding on a per-student basis.

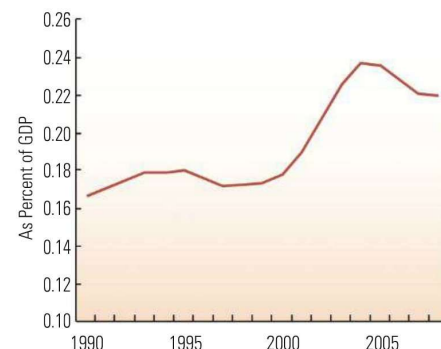
The report isn't solely about money, however. It says that state and federal rules governing the conduct of research should be more consistent and flexible, and some should be loosened. Examples of what the report calls “excessive regulatory burdens that put a drag on the efficiency of all university research” range from overly detailed accounting for a professor's time on a grant to the disposal of toxic materials. In return, the report says, universities need to shorten the length of time it takes students to earn their Ph.D., reduce attrition rates in graduate school, and trim administrative costs. The report cites actions by individual institutions and asserts that such cost savings “can be deployed to other priorities such as constraining tuition increases, increasing student financial aid, or launching new programs.”

There's also one revenue-neutral recommendation that is likely to rile rank-and-file scientists: Take some federal dollars that are now being spent on direct research and use them to more fully reimburse universities for the cost of supporting the research done on their campuses. A 2000 report estimates this shortfall in indirect costs at up to \$1.5 billion, and school administrators say that figure has grown along with the overall increase in federal funding.

The new report hopes to emulate the enviable record of an academies panel assembled in 2005. That response to a similar request from legislators for how to maintain U.S. global competitiveness produced *Rising Above the Gathering Storm*, a report whose recommendations formed the basis of a



Up or down? Federally funded research at U.S. universities more than tripled between 1990 and 2008. But in both the mid-1990s and the mid-2000s, the rise was offset by a booming economy that bent the curve.



2007 law authorizing increased funding for research and putting a greater emphasis on science and math education in the nation's elementary and secondary schools.

However, the world has changed a great deal since 2005, when the economy was booming and there was bipartisan support in Washington for what *Gathering Storm* would soon recommend. In addition to a protracted global recession and growing pressure to trim federal spending, politicians and the public are increasingly unhappy with the quality and value of a college education. That unhappiness has translated into demands that university administrators do a better job of

educating students and controlling costs—and that they be held accountable if their institutions fall short.

William Frist, who as a transplant surgeon and a former majority leader of the U.S. Senate has roots in both the political and scientific communities on the panel authoring last week's report, says he believes that a bad economy is exactly the right time to expand public support for research universities. "I can tell a governor who needs to balance his budget and boost his state's economy that the best way to do that is through strengthening his research universities. There's nothing more impor-

tant than growing the pie," says Frist, who retired from Congress in 2006.

Although the committee spent more than 2 years on the project, Charles Holliday, an engineer and former DuPont CEO who chaired the panel, said last week that "this is the start, not the end," of the committee's work. Panelists have agreed to host a series of regional workshops in the next year, followed by a national symposium in October 2013. The goal, Holliday says, is to reach consensus on "what we can do right now, without legislation, to make prosperity and security happen."

—JEFFREY MERVIS

ASTRONOMY

NASA's New X-ray Satellite Packs Compact Power

In theory, physicists know what happens to matter swirling around a black hole. As it gets closer to the black hole's event horizon—the boundary inside which the black hole's gravity exerts an inescapable pull—the matter gets sucked in. But what exactly happens to it? A new space telescope launched by NASA last week could help astronomers answer that and a host of other questions.

The Nuclear Spectroscopic Telescope Array—NuSTAR—will give researchers their best view yet of the universe in the high-energy x-ray band of the electromagnetic spectrum. As the world's first telescope to focus high-energy x-rays onto a camera, NuSTAR will capture images "10 times crisper and 100 times more sensitive" than other high-energy x-ray instruments can provide, says Fiona Harrison, a physicist at the California Institute of Technology in Pasadena and NuSTAR's principal investigator. "It will enable studying some of the hottest, densest, and most energetic phenomena in the universe."

NuSTAR's observing prowess comes from an innovative aspect of its design: a 10-meter-long mast that will extend out of the satellite once it is in orbit. At the tip of the mast are 133 layers of glass arranged to deflect high-energy x-rays into two digital cameras. The mast itself is made up of "thousands of moving parts stacked together like Tinkertoys," says William Craig, the mission's instrument manager. Engineers were planning to command the mast to deploy a week after the mission's launch on 13 June.

Mission managers say NuSTAR is designed to deliver ambitious science at relatively low cost. For example, NuSTAR has the same focal length as Chandra and XMM-Newton—two low-energy x-ray observato-



Extendable. NuSTAR's mast will enable it to focus high-energy x-rays.

ries currently in orbit—but is just a fifth of their size. (Having a long distance between the light-collecting instruments and the detectors—that is, a focal length of several meters—is key to getting good resolution.) As a result, NASA was able to launch NuSTAR on a small Pegasus rocket, limiting the mission cost to \$180 million. Harrison expects the observatory's deployable optics to become an example for future missions.

The primary targets for NuSTAR's observations will be black holes in the Milky Way. Matter in the immediate vicinity of a black hole gets heated to very high temperatures, emitting high-energy x-rays that cannot be detected by existing instruments. "These high-energy x-rays can tell us a lot about the physics of these regions immediately surrounding a black hole," Harrison says. "For example, by looking at these x-rays, you can determine the temperature of the hot electrons swirling around the black hole." By combining NuSTAR's data with low-energy x-ray observations from Chandra and other

observatories, she says, astronomers will be able to model what's going on near the black hole's event horizon. These studies will ultimately help researchers better understand the relationship between the growth of galaxies and the mass of black holes at their centers.

NuSTAR will also help discover black holes that are shrouded by dust and gas. "Low-energy x-rays get absorbed by the dust," Harrison says, but high-energy x-rays can shine through. Harrison and her colleagues also hope to point NuSTAR at supernova remnants to probe how supernovas explode, and to study gamma-ray bursts and other energetic expulsions from supermassive black holes.

Harvey Tananbaum, a Harvard University astrophysicist who is the principal investigator of Chandra, says that in addition to making "major advances" by itself, NuSTAR will scout out likely targets for Chandra and other low-energy x-ray telescopes to probe for new discoveries.

—YUDHIJIT BHATTACHARJEE

CREDIT: NASA/JPL-CALTECH

INFECTIOUS DISEASES

Second Bacterium Theory Stirs Haiti's Cholera Controversy

A just-published study is set to reignite the politically sensitive debate about the origins of the cholera epidemic that has killed some 7000 people in Haiti and sickened another half-million since 2010. The disease, which is still raging in the island nation, is widely thought to have been introduced to Haiti by United Nations peacekeepers, but some contest that verdict. "Our results show that the situation is much more complex and raise serious questions" about the idea that a single imported strain of the bacterium *Vibrio cholerae* caused the outbreak, says Rita Colwell of the University of Maryland, College Park, who led the new study.

Some scientists, however, are harshly criticizing the work and conclusions of the former director of the U.S. National Science Foundation. "In my opinion, this paper tries to obscure the published facts and invent controversy," says John Mekalanos, a molecular biologist at Harvard Medical School in Boston, who has studied the Haitian outbreak.

In January 2010, a devastating earthquake struck Haiti, killing a quarter of a million people. Nine months later, the cholera epidemic started, startling everyone because the disease had never been recorded in Haiti. From the beginning, some Haitians fingered U.N. peacekeepers from Nepal who were stationed close to where the first cases occurred. Colwell, a long-standing proponent of the idea that cholera outbreaks are usually caused by *V. cholerae* that lurk in natural reservoirs, disagreed, suggesting that environmental factors triggered an indigenous strain into causing the epidemic.

Colwell's position was largely dismissed, however, after many researchers compared the *V. cholerae* samples found in Haitians with samples from other countries. One team sequenced the genomes of strains that had been circulating in Nepal shortly before the peacekeepers left there and found them to be almost identical to the Haitian strain, which is known as serogroup O1. In May 2011, a report to U.N. Secretary-General Ban Ki-moon concluded that evidence pointed "overwhelmingly" to an introduction of the epidemic-causing strain.

In her team's new work, Colwell presents evidence that an indigenous Haitian strain was involved in the outbreak, as well. She and her colleagues examined stool sam-

ples taken at the beginning of the epidemic from 81 people from 18 towns across Haiti who showed symptoms of cholera. In half of them, the researchers found the O1 strain that was isolated by many other scientists. It is one of two serogroups (the other is O139) known to cause cholera epidemics. But in the stool samples of 17 patients, Colwell's team found a different strain of *V. cholerae* that was neither O1 nor O139; six of the patients with the O1 strain were also coinfecting with this strain. "Its role in this epidemic, either alone or in concert with *V. cholerae* O1, cannot be dismissed," the group writes in a paper published online this week in the *Proceedings of the National Academy of Sciences*.

Such non-O1/O139 *V. cholerae* bacteria are also found in U.S. coastal waters, and Colwell contends that the strains, which are known to cause diarrhea but not full-blown cholera, are widely distributed in estuaries

of the Nepalese and Haitian strains, says the frequency of non-O1 strains is surprising and that more research is needed "to understand its importance in this very large cholera outbreak." Yet Keim cautions that the new study "doesn't really help with understanding the origins of the O1 strain" found in Haiti.

Epidemiologist Renaud Piarroux of the University of the Mediterranean in Marseille, France, whose data pointed to the U.N. peacekeepers as a source of the epidemic, is not surprised that the O1 strains of *V. cholerae* showed up in only half of the people diagnosed with cholera. "That is not unusual. We often only find the pathogen in 50% to 60% of the cases," he says, in part because some patients with diarrhea likely are actually suffering from another pathogen, such as a virus. Colwell agrees that she cannot rule out a virus, "but it is a bit of a stretch to say that just because we didn't find O1 in these patients that came in to be treated for cholera, they didn't have cholera."

Ralph Frerichs, an epidemiologist at the University of California, Los Angeles, says that non-O1/O139 strains of *V. cholerae* had not been found in Haiti before, "but that does not mean that that is what started

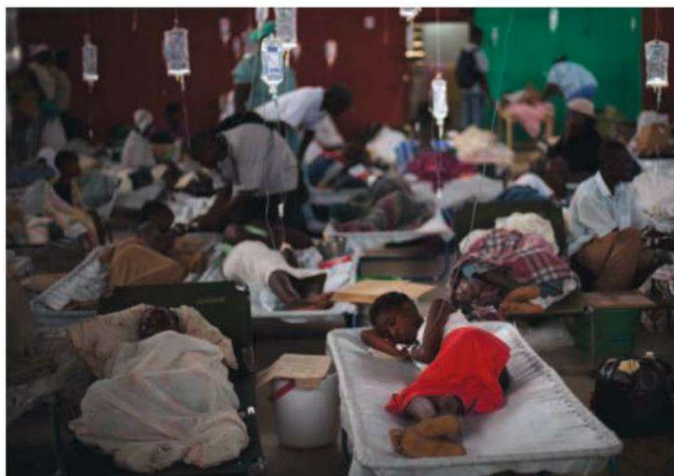
the vast epidemic there." Although Colwell carefully avoids claiming that, she insists that it has not been irrefutably shown that the cholera was introduced to Haiti.

The "single biggest flaw" in the paper, Mekalanos says, is that Colwell's team did not compare the genomes of the strains they found to the genome sequence of the 2010 Nepal isolates. Colwell says she has made that

comparison but left the results out deliberately and is preparing a separate paper. "Otherwise, the only focus would have been on the similarity of the strains. And this important point, that there is another strain of *V. cholera* [circulating in Haiti], would have been lost," she argues.

David Sack, an epidemiologist at Johns Hopkins University in Baltimore, Maryland, thinks Colwell has a point. "I suspect that all the pieces needed to explain the epidemic in Haiti have not yet been fit together."

—KAI KUPFERSCHMIDT



Unexpected epidemic. Cholera had never been reported from Haiti before the outbreak in 2010 sickened hundreds of thousands of people.

and rivers worldwide. In their study, she and her colleagues report finding such a strain in all water samples taken from two towns in Haiti. Adding up the evidence, Colwell proposes that the 2010 earthquake, in conjunction with Haiti's unusually hot summer, flooding from a hurricane, and poor sanitation, created a "perfect storm" of conditions ideal for the normally milder pathogen to contribute to a deadly outbreak.

Paul Keim, a microbiologist at Northern Arizona University in Flagstaff who was part of the team that sequenced the genomes

AVIAN INFLUENZA

Public at Last, H5N1 Study Offers Insight Into Virus's Possible Path to Pandemic

Depending on your point of view, the study that appears on page 1534 of this issue of *Science* marks another good week for public health experts trying to protect a vulnerable world from a new influenza pandemic—or for future bioterrorists bent on unleashing one.

The paper, from a laboratory led by virologist Ron Fouchier of Erasmus MC in Rotterdam, the Netherlands, describes how a handful of mutations might give the H5N1 avian influenza virus, which typically infects birds, the potential to move easily between mammals and touch off a human flu pandemic. It appears after more than 8 months of often fierce international debate over whether the results should be made public—and whether researchers should have conducted the experiments at all.

Late last year, the U.S. National Science Advisory Board for Biosecurity (NSABB) unanimously asked *Science* not to publish the study's details. (The journal agreed, in principle.) But in March, the same board voted 12 to six in favor of full publication after reviewing a revised and extended version of the manuscript and other evidence (*Science*, 6 April, p. 19). Along the way, the debate prompted influenza scientists to self-impose a landmark moratorium on some types of H5N1 research (see p. 1496), the U.S. government to set new controls on taxpayer-funded studies involving potentially dangerous pathogens, and the Dutch government to consider blocking publication by invoking export-control laws.

The paper is the second one in 2 months to suggest that H5N1 has pandemic potential.

Last month, *Nature* published a similar study by Yoshihiro Kawaoka of the University of Wisconsin, Madison, and the University of Tokyo that was also caught up in the controversy (*Science*, 4 May, p. 529).

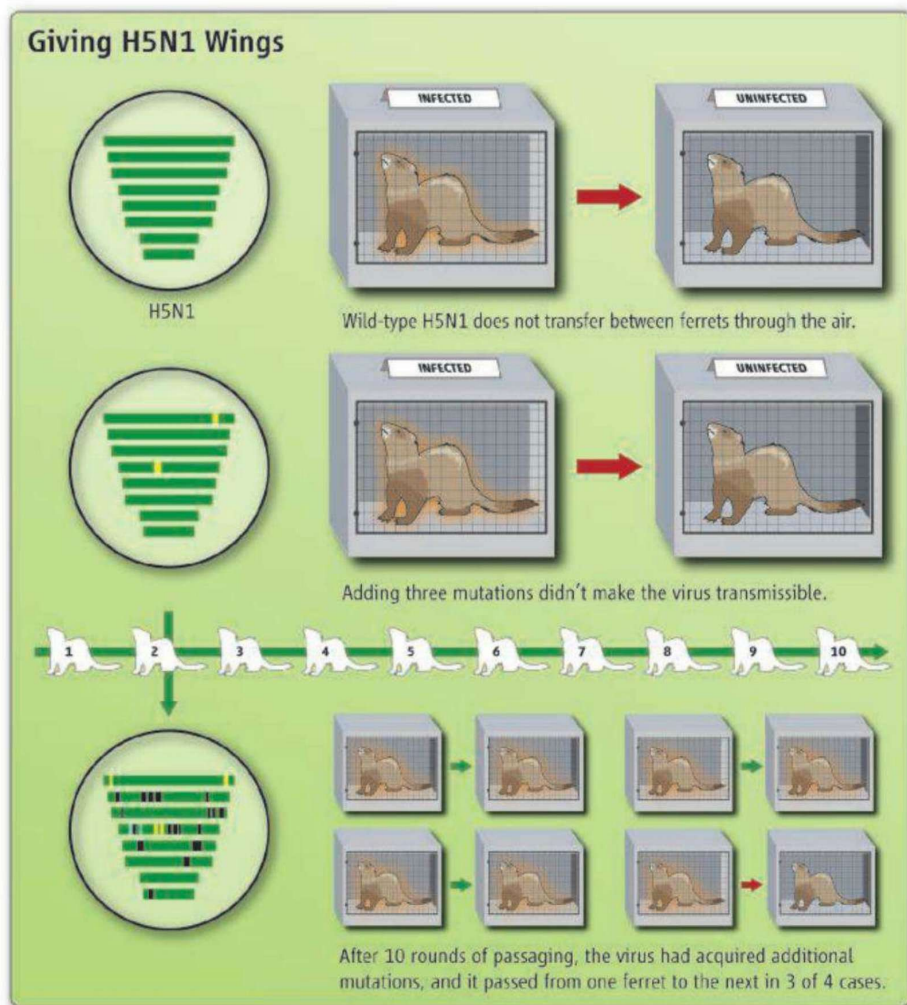
Until now, Fouchier had publicly discussed his study in only very general terms, including in a talk at a September 2011 flu meeting in Malta that triggered wide media coverage. Seeing the data in full is “sobering,” says influenza expert Nancy Cox of the U.S. Centers for Disease Control and Prevention in Atlanta, because it suggests that it's easier for H5N1 to trigger a pandemic than other studies—including her own—had indicated. In combination with the Kawaoka paper, Fouchier's findings shed light on how the virus could become pandemic, says Malik Peiris of the University of Hong Kong, and how public health officials might watch for mutations that could send it on its way.

Although it has decimated poultry flocks and killed more than 600 people since it first surfaced in 1997, H5N1 has not touched off a pandemic in humans because it hardly ever spreads from one person to the next—and some scientists think it never will. To become pandemic, the virus would have to become “airborne,” or able to spread via tiny droplets spewed out during coughing or sneezing. That is how other influenza strains spread among humans, and both Fouchier and Kawaoka wanted to know which mutations might allow H5N1 to do the same.

There's a key difference between the studies, however. Kawaoka created a hybrid virus: He took the gene for a viral protein called hemagglutinin from an avian H5N1 strain and stitched it together with seven other gene segments from the pandemic H1N1 virus that swept the world in 2009 and 2010, and which is already well-adapted to humans. From this starting point, it took just four mutations in the hemagglutinin gene to create a virus that could travel through the air from one infected ferret—a popular animal model for human infection—and infect another. But Kawaoka's hybrid has not yet been found in nature.

In contrast, “the strong point” of Fouchier's study, Cox says, is that it started out with an actual H5N1 virus isolated from a human victim in Indonesia. In an e-mail to *Science*, Kawaoka agreed that Fouchier's study addresses the most urgent question more directly. “Ron's data are very important,” he said.

Fouchier's team first inserted several mutations they knew might help the virus adapt for mammalian spread. One key target was the virus's receptor binding site, the area within the hemagglutinin molecule



For Young Scientists, A Wild Ride

ROTTERDAM, THE NETHERLANDS—They've become known as the Kawaoka and Fouchier papers. But Yoshihiro Kawaoka and Ron Fouchier rarely set foot in the high-security labs where the experimental work on their two highly controversial H5N1 studies was done. That work—concocting mutant viruses, inoculating ferrets, and testing whether they'd infect others—was carried out by younger researchers who have remained invisible during the past 8 months. Yet for them, the stakes were just as high—higher, perhaps, because a paper in *Science* or *Nature* can be a critical career booster.

Earlier this week, Sander Herfst, a virology postdoc at Erasmus MC here and the first author of Fouchier's paper, published on page 1534 of this issue of *Science*, was getting the champagne ready and announcing a lab party on the bulletin boards. "We're incredibly happy," he says.

While Fouchier gave interviews, traveled to meetings, and lobbied to get his paper published, Herfst stayed in the background, as did Ph.D. students Eefje Schrauwen and Martin Linster, the second and third authors, respectively. Once the National Science Advisory Board for Biosecurity (NSABB) got involved, "it was clear that this was being discussed at a level where we didn't belong," Schrauwen says.

The same was happening at the University of Wisconsin, Madison, where foot soldiers in Kawaoka's lab spent months waiting and worrying whether their paper would ever get published. Masaki Imai, the first author, wrote in an e-mail to *Science*. (*Nature* finally published it last month.) Imai, who is Japanese, obtained a Ph.D. at the University of Hokkaido in Japan, as did the second and third authors, Tokiko Watanabe and Masato Hatta.

In both studies, it was these bench scientists who saw the first signs that they had created new strains of the H5N1 virus that were transmissible from one ferret to another through sneezing and coughing—a finding they realized would be huge news. In Rotterdam, it happened in late June 2011, when a PCR test suggested that a ferret housed in a cage adjacent to an infected one had traces of the H5N1 virus in its airways. "We were very excited," Herfst says. "When we showed it to Ron, he just said: 'Calm down, and do it again. It may be an error.'"

It wasn't. But while he expected to make headlines, Herfst says he never imagined that the paper would get a red light from the NSABB and become the focus of a heated international debate about the limits of



In the background. Sander Herfst, Eefje Schrauwen, and Martin Linster did the hands-on work in Ron Fouchier's laboratory in the Netherlands.

academic freedom. Watching the flood of news coverage on TV, "it was strange to think that we had created all of that in our lab," Schrauwen says. "I thought that people would understand how important this kind of work is," Watanabe wrote.

The issue dominated lunch breaks at the lab but began to surface in private conversations, as well. A friend who had read the news stories but didn't know Herfst was involved warned him to watch out "because they are doing some pretty dangerous things at Erasmus." Others asked critical questions: Was this study really necessary? Linster says he could usually convince them. "Debates about animal experiments are more difficult," he says.

Members of both teams, however, worry that the controversy may deter budding scientists from entering the field. "They might be afraid or feel anxious or apprehensive about rejection of papers as a result of biosecurity concerns," Imai wrote. Indeed, a postdoc planning to come to Rotterdam won't work on H5N1, Herfst says. But Hatta thinks it won't be a problem. "Seeking the truth is the job of the scientists. I think nothing affects their motivations," he wrote.

Now that both papers are published, Herfst hopes the moratorium on H5N1 transmissibility studies will be lifted soon (see p. 1496). "We have a long list of interesting things we'd like to do," he says. But Herfst and his colleagues realize that that debate, too, is held well above their pay grade.

—M.E.

that makes first contact with the host cell; scientists already knew that two mutations there can make the virus prefer mammalian cells over bird cells. Another mutation, in the polymerase protein complex, allows the virus to replicate in the cool environment of the human upper respiratory tract rather than in bird intestines, the much warmer environment where it usually resides.

These initial mutations alone didn't do the trick, however, so Fouchier's team decided to try a time-honored method to encourage a pathogen to adapt to a new host: They passed the virus from ferret to ferret by directly inoculating uninfected animals with nasal samples from infected ones and repeated the procedure

a total of 10 times. (In his Malta talk, Fouchier called this a "really stupid" approach, a phrase widely interpreted to mean he regretted it. In fact, he says, he just meant that the technique, called passaging, is a simple one compared to the sophistication of creating targeted mutations. The confusion may have stemmed in part from the fact that the Dutch word for "stupid" can also mean "simple.")

The end result was a virus that could move through the air from one caged ferret to another right next to it; in a first experiment, the virus transmitted from cage to cage in three out of four instances.

Prior to publication, media reports suggested that airborne transmission required

five mutations. The reality is more complex. Each of Fouchier's transmissible viruses had at least nine mutations, five of which were shared by all. This core quintet may be sufficient, the team writes, but the big question is whether one or more of the other changes also plays an important role, Peiris says.

Fouchier already knows part of the answer. Once his team achieved transmission in the summer of 2011, the researchers began additional experiments to identify the minimum set of mutations needed to make the virus airborne. But before those experiments were finished, they submitted their manuscript to *Science*, worrying that Kawaoka or other scientists might beat them

How Much Longer Will Moratorium Last?

When will it end? That's what many influenza researchers want to know about the landmark self-imposed moratorium on certain experiments on the H5N1 avian influenza virus that they agreed to earlier this year. The short answer: Who knows?

Initially, the 39 researchers who announced the moratorium on 20 January said it would last just 60 days (*Science*, 27 January, p. 387). But in February, the loosely organized coalition agreed to an indefinite extension to give experts and the public more time to discuss and address concerns about the safety and wisdom of experiments that could alter H5N1 in ways that make the virus more dangerous to humans. (Other H5N1 research, such as the testing of newly detected strains, continued.)

Now, some of the moratorium's signers are eager for research to resume. But many say they are perplexed about how that decision will be reached and who will decide. "I wish I knew how it was going to be resolved," says virologist Robert Webster of St. Jude Children's Research Hospital in Memphis, Tennessee, a moratorium signer who was deeply involved in the flu papers controversy. "We haven't discussed this," says virologist Yi Guan of the University of Hong Kong.

Some key players, meanwhile, predict it will be months before the standstill ends. "We've still got a lot of homework to do ... and some boxes to check" before the moratorium should be lifted, believes Anthony Fauci, the head of the National Institute of Allergy and Infectious Diseases (NIAID), which funded the controversial studies by Ron Fouchier of Erasmus MC in

Rotterdam, the Netherlands, and Yoshihiro Kawaoka of the University of Wisconsin, Madison, and the University of Tokyo. Although Fauci isn't a signer of the moratorium, he played an influential role in encouraging Fouchier, Kawaoka, and other leading influenza researchers to organize it.

The researchers reluctantly agreed, driven in part by warnings that governments, reacting to public fears and media reports of "doomsday" viruses, might clamp down on the field if scientists didn't act on their own. Some dubbed the move "Asilomar 2," a reference to the historic 1975 agreement among recombinant DNA researchers that halted experiments in their emerging field until safety guidelines were established.

Before the current moratorium can end, several things have to happen, according to moratorium signers, Fauci, and others:

- The U.S. government must release for public comment a document that explains how universities and private laboratories can help federal funding agencies screen proposed research projects for "dual use research of concern" (DURC) that could be used for good or nefarious purposes. The goal of the new DURC screening program, which was announced in late March and covers 15 "high risk" pathogens including H5N1, is to spot problematic studies before they begin. The document—which is expected to run to nearly 30 pages and will be accompanied by a 100-page background—could be released "sometime this summer," Fauci says.

- Scientists and funders will need to agree on which lines of H5N1 research are—and are not—worth the risks. Particularly problematic, say Fauci and others, are "gain of function" studies, such as Kawaoka's and Fouchier's, in which researchers create mutant viruses that gain capabili-

ties to the punch. "Usually when you discover something important, somebody else is discovering it, too," Fouchier says. (He was right: Kawaoka, who says he didn't know about Fouchier's work, had submitted his manuscript less than 2 weeks earlier.) Now, Fouchier declines to discuss the results from the additional experiments, which are on hold as a result of the moratorium.

The published paper shows that the core set of five mutations includes the three that the team introduced themselves and two more that arose during passaging. And the resemblance to what Kawaoka found is "quite remarkable," says James Paulson,

a glyco biologist at the Scripps Research Institute in San Diego, California. Both teams found that two mutations at the receptor binding site—one of them identical in the two studies—are important; both discovered an additional mutation that makes hemagglutinin lose a sugar group, which apparently helps make room for the mammalian host cell receptor. Kawaoka also found a mutation in the hemagglutinin's stalk that improves the virus's stability and compensates for the other mutations, Paulson says. "It's tempting to think" that one of Fouchier's mutations plays a similar role—although it's not in the stalk but

in another area where three hemagglutinin molecules align to form a so-called trimer.

Cox says she's "surprised" that it didn't take more mutations. In a study with Paulson published online by *Virology* in November, her team also mutated the receptor-binding site, but they had to make several other changes—including slotting in a human-adapted version of another viral gene called *neuraminidase*—to get airborne transmission. That led the authors to conclude that the virus might require "extensive evolution" to become pandemic. Fouchier's paper upsets that reassuring notion.

Both papers will aid surveillance efforts because they suggest which genetic changes to look out for in H5N1, Peiris says. But they also point to a limitation: Several mutations can have the same effect on the virus. "It wouldn't be appropriate to focus just on these mutations," Peiris says. "The virus has different ways to go from A to B." More research is needed to discover how many ways, he says.

In a second paper published this week and co-authored by Kawaoka and Fouchier (p. 1541), a group led by mathematician Derek Smith of the University of Cambridge in the United Kingdom takes a stab at understanding the likelihood of the emergence of a pandemic H5N1 strain. The researchers first combed through surveillance databases to determine whether the mutations identified in the two controversial studies have already appeared in



Nothing to sneeze at. The study tested for airborne transmission of H5N1 by placing infected and uninfected ferrets in adjoining cages.

CREDIT: PHOTO COURTESY OF S. HERBST

At a standstill. Certain experiments with H5N1 are on hold until signers of a self-imposed moratorium, including Yoshihiro Kawaoka (left) and Ron Fouchier (right), agree to lift it.

ties—such as mammalian transmission—that naturally occurring versions do not have. A key step in this process could come in late July, when the heads of NIAID-funded influenza laboratories are scheduled to meet in New York City.

- Laboratory safety officials and scientists will need to “at least have a consensus on the level of biocontainment required” for H5N1 studies, says microbiologist Adolfo García-Sastre of Mount Sinai School of Medicine in New York City, a leader of the moratorium. Currently, most H5N1 studies occur in biosafety level 3 (BSL-3) laboratories, but some critics argue that they should be restricted to a small number of higher-containment BSL-4 laboratories.

In the meantime, many signers say the moratorium has already achieved its goal. “The voluntary action ... helped calm people’s concerns so that discussion could take place,” Kawaoka says. And it “provided the time to deal with these issues in some depth,” says Thomas Mettenleiter of the Friedrich Loeffler Institute in Griefswald–Insel Riems, Germany, even though “no universal ‘solution’ was found.”

—DAVID MALAKOFF

With reporting by Martin Enserink.



nature. They found that many H5N1 isolates are three—and in a few rare cases, just two—mutations away from Kawaoka’s quartet and four from Fouchier’s quintet.

They then developed a model of viral evolution to test whether existing viruses, once they happen to infect a mammalian host, might accumulate the missing mutations and become excreted in respiratory droplets, which could start a chain of transmission. The model takes into account a variety of factors, such as the duration of the infection and whether individual mutations by themselves benefit the virus. The conclusion, says first author Colin Russell of the University of Cambridge, is that a virus that is only three mutations away from the full set is “likely” to acquire them and end up in droplets. But the paper can’t put a number on that risk; there are too many unknowns.

“You can do a lot of fancy maths, but in the end the probability is hard to pin down,” Peiris says. Still, “it’s a model of how modeling should be done,” says Steven Wolinsky, who studies HIV evolution at Northwestern University in Chicago, Illinois. “They do a very nice job of explaining all the caveats.”

The study was presented both at a World Health Organization meeting about the papers in February and during the second NSABB review in March, and it helped convince a majority on the panel that H5N1’s risks were real enough to warrant publishing Fouchier’s

paper, says NSABB acting chair Paul Keim, a microbial geneticist at Northern Arizona University in Flagstaff.

Another factor that swayed the board to support publication, NSABB members say, is that the published version of Fouchier’s paper does a much better job of clarifying the lethality of his airborne virus than the first version they read. Fouchier says the draft he submitted to *Science* did not discuss whether his airborne viruses killed the ferrets they infected. But two of the three reviewers asked for additional experiments examining lethality, which Fouchier said took just a week to perform, “so we did them,” and added a line to the paper describing the outcome.

That language—combined with Fouchier’s remarks in interviews and sometimes hyperbolic press coverage—appeared to suggest that the airborne mutants were extremely lethal, which “greatly alarmed” many NSABB reviewers, says virologist Robert Webster of St. Jude Children’s Research Hospital in Memphis, Tennessee, an influenza expert who was asked to advise NSABB on the two papers. In fact, however, none of the ferrets had died from airborne transmission; six ferrets that had the virus squirted directly into their trachea all died. But that outcome is “not very relevant” for evaluating the virus’s risk, Fouchier notes, because that’s not how humans or animals typically contract flu. He says the manuscript

NSABB saw in its first review last year made clear that the different routes of infection led to different outcomes.

But Webster says the results were presented in a way that confused NSABB. He says that the experienced virologists involved in NSABB’s discussions—including himself—should have pushed harder to clarify those results and emphasize that lethality in ferrets does not necessarily predict lethality in humans. When the lethality data finally became clearer in the months after the NSABB’s initial recommendation, many members say they began to reconsider. In retrospect, Fouchier says, “we should have ignored [*Science*’s] reviewers’ request for lethality data,” given the confusion that ensued.

The publication of Fouchier’s paper isn’t likely to be the last word on such issues, however, especially as influenza researchers seek to restart similar studies now stalled by the moratorium. Many, however, are treading cautiously, eager to avoid replaying the drama of the last 8 months. Fouchier, for his part, says he’s “sick of all these discussions,” and he declined to release his first manuscript to reporters in order to help clarify how the story unfolded, a step Keim says he would support. “I want to move on,” Fouchier says. “Maybe in 5 or 10 years’ time, when someone writes a book about all of this.”

—MARTIN ENSERINK

With reporting by David Malakoff.

Hang On! Curiosity Is Plunging Onto Mars

The performance this August of a brand-new system for landing on Mars means life or death for NASA's next rover mission, but future Mars exploration hangs on a perfect touchdown as well

NASA'S VIDEO DEPICTING THE NEXT MARS rover's descent toward the martian surface makes some planetary scientists nervous (<http://scim.ag/rovervideo>). NASA has \$2.5 billion riding on the success of Curiosity's scary-new "sky-crane" landing system designed by engineers at NASA's Jet Propulsion Laboratory (JPL). If Curiosity crashes as it dangles by cables from its flying platform festooned with blazing rockets, some scientists will see a decade or more of their careers crash with it.

But there's even more riding on Curiosity's safe touchdown at 10:31 p.m. PDT this 5 August (5:31 UTC on 6 August). Not only will NASA have to slow the most massive load ever delivered to another planet's surface from hypervelocity bullet speeds to a dead stop, all in the usual "7 minutes of terror." But NASA is also attempting to deliver Curiosity to the surface of Mars more precisely than any mission before, within a 20-kilometer-long ellipse some 240 million kilometers from Earth. Both feats are essential to NASA's long-term goals at Mars: returning samples of martian rock and sending humans to the Red Planet.

"I'm very confident in our system," says Steven Sell of JPL, a lead engineer on the seven-person team responsible for Curiosity's atmospheric "entry, descent, and landing," or EDL. "We know [EDL] inside and out. I feel very confident we have done everything we can do to make it as reliable as possible." Still, he adds, "I'll be as nervous as anyone on landing day."

Online

sciencemag.org

S Podcast interview with author Richard A. Kerr (http://scim.ag/pod_6088).

Designing 7 minutes of terror

Six times before, NASA has managed to land spacecraft safely on Mars, starting with the two Viking landers in 1976. This time, NASA planners wanted a bigger and better—and, therefore, heavier—rover to advance the search for life. Curiosity, clamped inside its entry vehicle like the innards of a clam, weighs in at 3.3 tons—three times larger than any previous entry vehicle. Still, as in past missions, the job of safely braking the rover from 21,240 kilometers per hour falls to the drag of the wispy martian atmosphere. Starting at Mach 20, the bulletlike heat shield bears the brunt of the slowing (see figures, upper left panel). Then a lone parachute deploys at Mach 2 (upper right). To handle the greater mass, engineers

had to enlarge and beef up both the heat shield and the parachute. They expect these scaled-up "heritage" designs to get Curiosity through the fireball entry stage, the parachute phase of descent, and thus 98% of speed reduction.

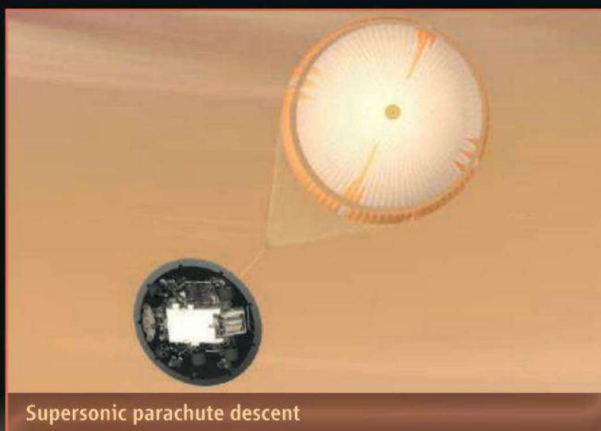
The next stage, though, presented a problem. Some earlier missions delivered immobile landers. In their final, powered descent stage, touchdowns like those of the Vikings had depended on easing the lander down on retrorockets to land on its own legs, as in 1950s sci-fi movies. The three mobile rovers have instead bounced onto Mars inside the NASA equivalent of beach balls. But landing the much bigger Curiosity on a legged, powered platform would be trickier, Sell says. The bigger lander would be less stable on uncertain slopes, and the whole system would be more sensitive to the critical timing of engine shutdown. And a beach ball just wasn't going to work. "The airbag system was as big as it could be made," he says. "It had played out."

So engineers brainstormed. "On Earth, how do we deliver big things?" Sell asks rhetorically. "On construction sites, they use cranes or helicopters. Thus, the rover-on-a-rope was born." Curiosity, its six wheels deployed, will be lowered 7.5 meters below its rocket-powered descent stage on three cables

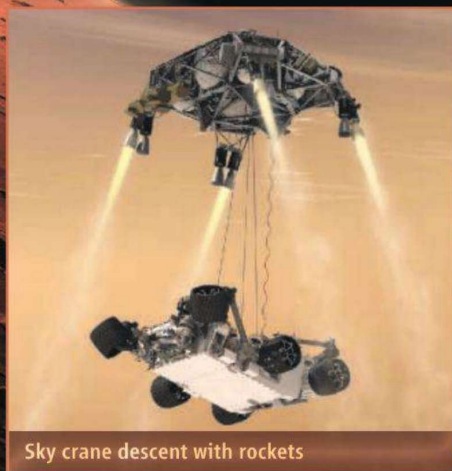
CREDIT: NASA/JPL-CALTECH



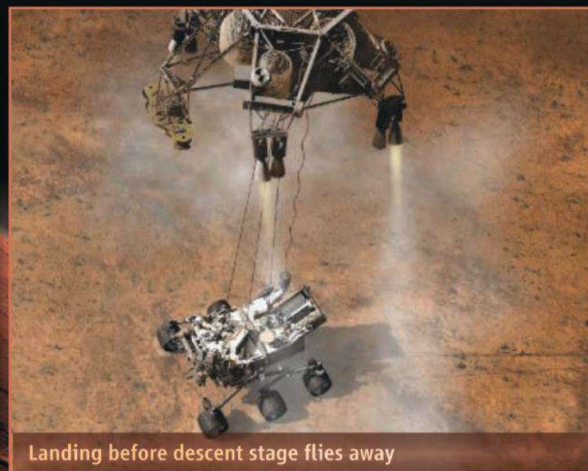
Fiery atmospheric entry



Supersonic parachute descent



Sky crane descent with rockets



Landing before descent stage flies away

Destination Gale. Curiosity rover is headed for 154-kilometer-wide Gale crater (left page with a central mound). Left to right from far left, landing safely will require first plowing through the atmosphere behind a blazing heat shield, then plummeting under a parachute. Rockets slow the dangling rover further in time for touchdown.

in the “sky crane” phase of descent (lower left panel). Once the rover eases onto the surface (lower right), the control system will sense the drop in tension on the cables and the descent stage will fly out of harm’s way.

Less innovative was the engineers’ solution to the problem of shrinking the rover’s landing zone from an ellipse 100 kilometers long to one just 20 kilometers long. Planners wanted to be able to land their bigger, better rover near the most interesting geology, whether or not there was a broad swath of forgiving terrain there. So Curiosity engineers borrowed the concept of “guided entry” from the system used for returning Apollo astronauts to Earth.

All previous Mars landers had entered the atmosphere like bullets; they could hit their targets no more accurately than they were aimed. Throw in the vagaries of entry vehicle performance and martian winds, and they arrived a good deal less accurately than that. The limited targeting accuracy of the previous generation of rovers kept them out of Gale crater, whose central mound boasts a Grand Canyon–like exposure of early Mars history.

With guided entry, Curiosity’s 3.3-ton bullet becomes more like a guided missile. Tilted slightly up so it can “surf” the martian atmosphere, the entry vehicle will sense departures

from its intended entry track. Then it will fire side thrusters to get back on track. The promised accuracy of guided entry let the Curiosity team, with community guidance, target the flat Gale crater floor within driving distance of the mound (*Science*, 29 July 2011, p. 508).

Testing, testing, testing

“That’s a lot of parts that have to work together,” as one scientist observed after watching the EDL video. So with 30 or 40 other engineers, the Curiosity EDL team “analyzed, peer-reviewed, and tested the hell out of” the EDL system, as Curiosity project manager Peter Theisinger of JPL put it during a press teleconference last week. The problem is that “you cannot test as you fly,” he noted. “It is impossible to replicate the EDL conditions of Mars.”

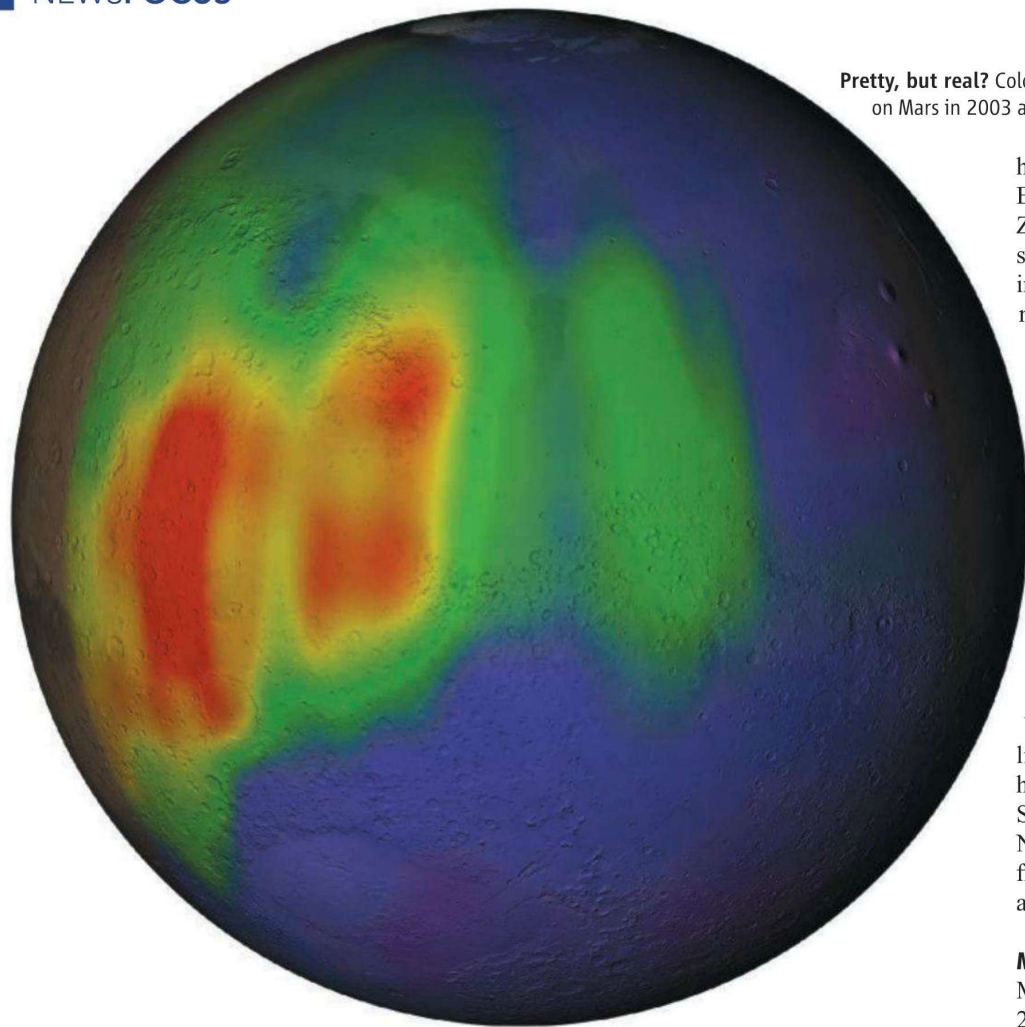
Instead, engineers first tested each EDL component as realistically as they could. They checked the strength of a full-size, 21-meter-diameter Curiosity parachute in the world’s largest wind tunnel, tested a scaled-down version for aerodynamic stability in another wind tunnel at realistic supersonic speeds, and investigated aerodynamic regimes inaccessible in wind tunnels in computer simulations. Then they

tweaked their design and tested again.

Entry-to-touchdown testing came in a computer simulation of “the entire spacecraft built in a computer,” Sell says. That let them “fly EDL over and over and over again. We do that with thousands of variables and run the system millions of times.” Such testing makes the EDL system “as robust as possible to variations at Mars,” he says, from a gust of wind to an underperforming retro-rocket. “We’re still doing it,” Sell says, and “we’ll keep doing it until landing day.”

“We’re confident we’ve done everything we know how to do,” Theisinger said. “You do the best testing you can on Earth, but it’s not the same. That’s where the trepidation is. It’s really the unknown unknowns we’ll be worried about on the 5th.” That’s what stung engineers in 1999 during Mars Polar Lander’s Viking-style EDL. Investigators suspect that the onboard control system mistook the jolt of the lander’s legs snapping into place for touchdown and cut the retrorockets while the lander was still 40 meters off the ground, ending the mission. Phoenix later landed using the same EDL system (with a software fix), but unknown unknowns are likely still in any EDL. As Sell puts it, “good or bad, August 5th we’re on Mars.”

—RICHARD A. KERR



Pretty, but real? Colors denote concentrations of atmospheric methane on Mars in 2003 as reported by Mumma and colleagues.

PLANETARY SCIENCE

Could a Whiff of Methane Revive The Exploration of Mars?

The controversy over claimed detections of martian methane—a possible product of life on Mars—could be settled come August once the Curiosity rover arrives at the Red Planet

How many cows are there on Mars? That depends on which planetary scientist you're asking. Some would say there have been thousands of head of cattle, judging by how much methane three independent groups reported detecting in the atmosphere of Mars in 2003. Earthly cows belch the digestive gas all day long, so a bovine equivalent for Mars is a convenient, if playful, way to quantify martian methane. Whether the reported methane is from microbes eking out a living beneath the surface or from deep stirrings of the geologically moribund planet, no one can say. Either would excite scientists, but only martian biology could rejuvenate a troubled NASA Mars program.

Despite the playful units, planetary researchers are quite serious about their meth-

ane on Mars. "I'm shocked by these results," said planetary scientist Michael Mumma of NASA's Goddard Space Flight Center in Greenbelt, Maryland, when he first announced his team's methane detection at a meeting in 2004, but "our results are certain." The researchers reported that they had detected a few tens of parts per billion (ppb) by volume of martian methane. They found it by reading the squiggly lines of infrared spectra recorded by ground-based telescopes. Two other independent groups backed up Mumma with their own reported spectroscopic detections.

But some planetary scientists now see no credible signs that there ever was any methane on Mars. Last October, planetary scientist Kevin Zahnle of NASA's Ames Research Center at Moffett Field in California called

his invited seminar on the subject "Lack of Evidence for Animal Husbandry on Mars." Zahnle has never done spectroscopy of any sort. But he and more spectroscopically inclined colleagues have scrutinized Mumma's results, noting that the reported parts-per-billion detection relied on instruments looking through the 2000-times-more-abundant methane of Earth's atmosphere. Correcting for that earthly methane "is just really, really hard," Zahnle says. "I don't think they can possibly do it."

The right head count of martian livestock could have multibillion-dollar implications for the exploration of Mars. NASA's Mars science program took a severe hit in President Barack Obama's 2013 budget request this past February (*Science*, 24 February, p. 900). But once before, in the 1990s, hints of ancient martian life—that time in a meteorite from Mars—helped resuscitate NASA's Mars program. So within weeks after its arrival this August, NASA's rover Curiosity could again fan the flames of life on Mars if it detects uncontested martian methane.

Methane, methane everywhere?

Martian methane burst on the scene in March 2004 at a press conference in Paris (*Science*, 26 March 2004, p. 1953), and was soon followed by two more independent claims in a meeting and its press conference. All three research groups reported at least 10 ppb of methane in the atmosphere of Mars in 2003. That's just 10 cubic centimeters of methane dispersed in the thousand cubic meters of a 10-meter cube of thin martian air. The three claimed detections—all since published in leading peer-reviewed journals—are still often cited as reinforcing one another, implying some measure of consensus. But they have not fared equally well among experts.

All three reported detections rely on recognizing methane's signature in the sun's infrared radiation reflected from the surface of Mars. As solar radiation passes through the martian atmosphere, methane molecules—carbon atoms studded with four hydrogen atoms—absorb infrared energy at specific wavelengths. A spectrometer's optics break the reflected radiation into a rainbowlike spectrum in which scientists can recognize the narrow "lines" of absorption unique to methane.

First to announce the detection of methane was Vittorio Formisano of the Institute of Physics and Interplanetary Space in Rome and the orbiting Mars Express spectrometer

CREDIT: TRENT SCHINDLER/NASA

team. After the announcement at that Paris press conference, Formisano told *Science* that he and his team “have seen methane on Mars. A very little amount, but the result is clear.” The group’s paper appeared in the 3 December 2004 issue of *Science* (p. 1758), but its reported detection of 10 ppb averaged across Mars has fared the worst of the three claims.

Sushil Atreya, an atmospheric chemist at the University of Michigan, Ann Arbor, and second author on the Mars Express paper in *Science*, says the Mars Express result was not a true “detection.” The spectrometer’s ability to separate methane’s absorption lines from interfering lines, its spectral resolution, was not up to snuff, he says, adding, “I would put more stock in ground-based” results.

The two ground-based claims of martian methane in fact look somewhat better. At times working with colleagues, planetary astronomer Vladimir Krasnopolsky of the Catholic University of America in Washington, D.C., has used spectrometers mounted on large telescopes to search for methane. In three papers in *Icarus*, the first in 2004, Krasnopolsky reported methane detections.

But Mumma, who has published with Krasnopolsky and supervised him at Goddard, has his doubts. Krasnopolsky’s first two reported detections suffered from technical problems in processing the data, Mumma says. In a third paper, in the January 2012 issue of *Icarus*, Krasnopolsky reports detecting 10 ppb of methane over the Valles Marineris canyon region of Mars in 2006. Mumma calls the result “interesting,” although his own group’s observations a few weeks before and after Krasnopolsky’s found no detectable methane over Valles Marineris.

The Cadillac of data reductions

By all accounts, Mumma’s observations have fared the best. For one thing, Mumma is highly regarded in the planetary observing community. He has measured 10 different volatile compounds, including methane, spewing from 30 active comets. Mumma and his group are also seen as having most thoroughly accounted for the bugaboo of ground-based observers: Earth’s own methane. An absorption line from Earth’s methane should be 2000 times as strong as a line from 20 ppb of methane on Mars would be. (After all, Earth has actual belching cows.)

Mumma first reported detection of martian methane at the annual meeting of the Division for Planetary Science (DPS) in November 2004. In his talk, Mumma reported finding 86 ppb of methane using one absorption line and 66 ppb using another line. That rough agreement from two independent

absorption lines remains the backbone of the Goddard group’s defense of their numbers. “The methane is secure,” he said. At a press briefing later the same day, however, he mentioned a methane concentration of 250 ppb. Over the next year or two, Mumma continued to report readings as high as 700 ppb, Atreya recalls. But none of the numbers appeared in DPS abstracts because Mumma considered them still preliminary.

After almost 4 years of increasingly thorough processing of the raw data, including accounting for terrestrial methane, Mumma and colleagues published their 2003 observations in the 20 February 2009 issue of *Science* (p. 1041). The group reported much lower final values for the 2003 methane detections—20 to 45 ppb—along with a detection of 5 ppb from 2006. Methane was concentrated in the vicinity of Syrtis Major and Nili Fossae near the equator and varied in abundance from season to season and year to year.

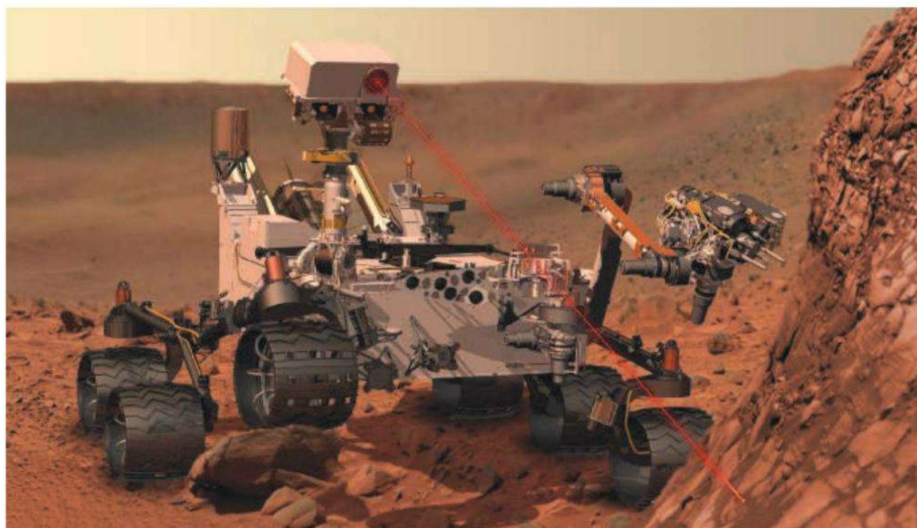
To Mumma’s group, that evanescent, plume-like behavior of methane suggested that shortly before the 2003 observations—and perhaps at other times—the planet had

the interference by observing Mars when it is moving toward or away from Earth. That’s when the Doppler effect shifts a given martian methane absorption line away from the same line created by terrestrial absorption, making the two lines distinguishable.

Mumma thinks that he and his co-authors—most of whom are at Goddard—have reliably accounted for any remaining methane interference using a sophisticated model of Earth’s atmosphere. The 20-person spectroscopy group he directs at Goddard is “a powerhouse in the world community,” he says. That view is widely shared. “Mumma has given [his data] about as good a look as can be,” says atmospheric chemist Paul Wennberg of the California Institute of Technology (Caltech) in Pasadena.

Not so fast

Zahnle wasn’t having any of it. With 30 years of experience modeling the atmospheric chemistry of the planets, he “just couldn’t imagine anyone taking [methane on Mars] seriously,” he says. “Methane doesn’t behave like this. It was such an obvious joke.



Methane sniffer. Before the Curiosity rover samples any rocks with its arm-mounted drill or rock-zapping laser, it will test the air for signs of methane, a possible product of any martian life.

been spewing a couple of tons of methane an hour from the ground. The methane might have been microbial, trapped kilometers beneath the frozen crust and released occasionally through crustal cracks. Or it could have been geological, escaping from volcanic or geothermal vents. No one can say what the source would have been, and no one has reported detecting any methane since 2006.

Key to determining whether the martian methane plumes were real is the procedure for removing the spectral signal of Earth’s methane. Ground-based observers avoid most of

I was upset that the community wasn’t taking an interest” in critically evaluating detection claims. Instead, methane claims were propelling a proposed orbital mission to Mars, NASA’s Trace Gas Observer, on its way to launching in 2016.

So Zahnle looked at methane on Mars the best way he knew how: from a chemical perspective. He concluded that the proposed scenario—repeated injections of methane into the atmosphere, followed by the methane’s rapid destruction—would wreak havoc on the composition of the martian atmosphere.



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As Zahnle lays out in an April 2011 *Icarus* paper, destroying that much methane that quickly would consume the atmosphere's meager endowment of oxygen in 7000 years. That might not happen if exotic processes had been overlooked—say, chemical oxidizing agents generated on the dust of self-electrifying dust devils.

But that doesn't seem to be the case, Zahnle says. The standard model of how solar energy creates and destroys chemical compounds already neatly explains the trace gas composition of the martian atmosphere without invoking any such hidden methane-busters. And nonphotochemical means of removing methane, such as adsorbing it onto soil, don't work either if theory, lab experimentation, and the trace gas composition of Mars are any indication, he says. Even microbes feeding on the methane probably couldn't cause the proposed disappearing plumes, Zahnle says. That's because the martian atmosphere abounds in carbon monoxide—a molecule that microorganisms with anything like earthly metabolisms would finish off before starting to consume methane.

Zahnle concludes that there is no support for claims that martian methane plumes were popping up repeatedly almost a decade ago. However, he can't exclude the possibility that a single plume appeared in 2003. "If it's one time in 100 years, it's not a problem," he says. That relieves atmospheric chemists' great unease with the idea of methane coming and going on Mars. "The photochemical argument Kevin made seems to be pretty solid," Atreya says.

About that spectroscopy

Next, Zahnle looked for possible problems in Mumma's spectroscopic methane detection. As an atmospheric chemist, he needed lots of help. So he brought in Richard Freedman of Ames, an astrophysicist and stellar spectroscopist, and David Catling of the University of Washington, Seattle, "somebody who knows something about Mars."

The trio found several possible problems with both the space- and ground-based spectroscopy, as they reported in the *Icarus* paper. But the focus of their criticism became the two absorption lines at the core of Mumma and colleagues' reported detections. Mumma, they note, is relying on only two methane lines and can see only one of them on any given observing night.

More worrisome, they say, is their suspicion that the same Doppler shift that conveniently brings Mars methane lines into view, by moving them away from Earth's methane lines, also moves a Mars line to where

a minor Earth methane line can impersonate the Mars line. The Mumma group has never seen methane when Mars is moving away from Earth; it is only seen when Mars is moving toward Earth, and the lines are shifted toward the blue end of the spectrum. Zahnle and colleagues suggest that in such a blue shift, the line of methane that contains the heavy carbon-13 isotope comes into coincidence with the martian line of normal methane. The heavy-carbon methane line is about 25 times as strong as the line of martian methane at 20 ppb would be. So the Earth line, the group contends, could appear to signify abundant methane on Mars.

"I'm no spectroscopist, but it's going to be challenging to correct for that," Zahnle says. "The point is not to slam Mike Mumma. This is just really, really hard; it's ridiculously difficult."

Mumma disagrees. "I respect Kevin as a colleague," he says, but "there are so many



Methanogenic? Water-altered Nili Fossae (color-coded by mineral) may have gushed methane in 2003.

errors" in the Zahnle *et al.* paper, some of which "were egregious." He points to three sorts of consistent behavior in the spectroscopic data that support martian methane. One involves the nonappearance of the terrestrial carbon-13 methane line when, as he sees it, Zahnle's reasoning would have it there. "We've got carbon-13 methane modeled correctly," Mumma says.

All this debate, which has not gotten much of a public airing, has left the larger planetary community a bit adrift. With 50 years working in the Mars community, geologist Michael Carr of the U.S. Geological Survey in Menlo Park, California, finds methane on Mars "such an iffy thing." John Mustard of Brown Univer-

sity does spectroscopy of the martian surface from orbiters. "It looks really problematic for Mumma's [detections]," he says. All the variability "just doesn't make sense. I'm thinking it's not there."

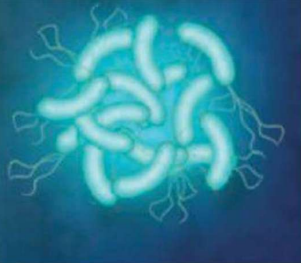
David Crisp of NASA's Jet Propulsion Laboratory (JPL) in Pasadena, California, does atmospheric spectroscopy. "My intuition is there's methane there," he says, "but it's not clear [Mumma] is seeing time and spatial variations." Interfering atmospheric dust is just too variable on Mars, he says. "If anything, I would trust the detection of methane more than the [reported] absolute amount," says Mark Allen of Caltech, who was the principal investigator of the now-canceled Trace Gas Orbiter mission. Mumma "is really doing differences of [two] large numbers to get a small number," Allen says, always a tricky bit of arithmetic.

Last chance?

Confirming such contentious claims will require getting up close and personal with Mars. The Trace Gas Orbiter was going to carry a capable spectrometer that "could detect three cows on Mars," Allen says. But NASA killed the mission when it restarted its Mars program in the wake of the Obama Administration's lowered budget request. Next up is NASA's Curiosity rover, arriving at Mars on 6 August. It carries a different sort of spectrometer, the Tunable Laser Spectrometer (TLS). It has two lasers whose beams bounce back and forth 81 times through a sample cell filled with martian atmosphere. The lasers scan across infrared wavelengths in which methane has not one or two but three absorption lines that form a distinctive fingerprint for methane. "It's a very simple, direct, and unambiguous measurement," says TLS developer Christopher Webster of JPL.

TLS will also be extremely sensitive. Its first quick measurement, scheduled for the first few weeks of the mission, should be able to detect as little as 1 ppb of methane. Mumma calculated that the 2003 release of methane would have amounted to 6 ppb once it spread around the planet. Although Mumma sees his observations as suggesting that methane on Mars has a lifetime of a few years, atmospheric chemists still think martian methane would last several centuries. If they are right, almost all of 6-ppb methane should still be there. And even if it is destroyed faster, when TLS takes a longer look at atmospheric samples, it will be able to detect 50 to 100 parts per trillion of methane, according to Webster. If methane really is long-lived on Mars, Zahnle says, Curiosity "is going to crush this."

—RICHARD A. KERR



LETTERS

edited by Jennifer Sills

Community Colleges: Veterans' Best Bet

AS J. C. MINER POINTS OUT IN HER EDITORIAL "AMERICA'S COMMUNITY COLLEGES" (23 MARCH, p. 1409), community colleges offer outstanding training opportunities in science, technology, engineering, and mathematics (STEM) for underrepresented populations. They also provide an important STEM training opportunity for veterans returning from Iraq and Afghanistan, a population that unfortunately has been preyed upon by for-profit universities.

The Post-9/11 G.I. Bill provides veterans and their families generous educational benefits but, as the U.S. Senate's HELP Committee's ongoing investigation reported (*1*), 37% of the \$4.4 billion spent between 2009 and 2011 went to for-profit educational institutions, which trained only 25% of the veterans. In these schools, the cost per veteran averaged \$10,875, compared with \$4,874 in public colleges. Moreover, the withdrawal rate for bachelor's students

in the eight most generously funded for-profit schools averaged a disappointing 53.3%, compared with 19.8% in public schools. The investigation reported that "[t]he majority of students enrolling in for-profit schools emerge with debt but without a diploma." President Obama sought to end these abuses through an Executive Order signed at Fort Stewart on 27 April (*2*).

Community colleges offer broad-based curricula, quality teaching, flexible class schedules, and (unlike many for-profit schools) college credits transferable to 4-year institutions, all at an affordable price.

Yet cuts in state funding are diminishing our community colleges. For example, California's outstanding system will endure a 5% budget cut in 2013, forcing a 20% decrease in course offerings (*3*); California's state college system is planning to freeze admissions, which will clog community colleges and curtail opportunities for new students, including veterans.

Many young people opt for military service specifically to qualify for educational benefits, and some are primed for STEM training because of the positions they filled in the service. We owe these students quality STEM educational opportunities. Community colleges offer a superior product and value; they are veterans' best bet.

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An Eye Toward Iodine in China

I WAS GLAD TO SEE THE NEWS & ANALYSIS story calling attention to China's continuing problem of rural malnutrition and resultant developmental stunting of children ("Despite gains, malnutrition among China's rural poor sparks concern," R. Stone, 27 April, p. 402). Two specific aspects of the problem bear emphasis.

First, iodine deficiency has historically played a dominant role in both mental and physical stunting in Chinese rural populations. Children born after the introduction of iodine supplementation (without other nutritional intervention) have lower infant mortality and improved development, activity, and stature (*1, 2*). Second, micronutrient supplementation, particularly iodine, should be administered during early pregnancy. Studies carried out in Xinjiang Province and in Inner Mongolia by our joint Chinese-American team demonstrated that iodine supplementation alone to pregnant women in areas of severe iodine deficiency, before the end of the second trimester, resulted in decreased infant mortality and in significantly improved development and stature in the offspring at 2 and 6 years of age (*3, 4*). Iodine supplementation at later times was progressively less effective.

Iodine deficiency continues to be severe in Chinese soils in many areas. In addition to other nutritional interventions, careful monitoring of iodine availability to the population, especially to women of child-bearing age and to young children, will need to be carried out indefinitely and with a high priority. A trial program of iodization



Supporting the troops. President Obama signs an Executive Order to protect veterans from deceptive marketing by for-profit schools.

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of irrigation water was successful in Xinjiang and Inner Mongolia, providing iodine to the entire population as well as to livestock within a given area (5, 6).

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Chile's Research Planning Aims High

IN THEIR LETTER "CHILE'S RESEARCH PLANNING falls short" (27 April, p. 412), P. Astudillo *et al.* argue that CONICYT—Chile's government funding agency for science and technology (S&T)—is insufficient and should be replaced by a ministry of science. In fact, it

is a good time to be a scientist in Chile.

Most of the data Astudillo *et al.* cite were collected before 2010. The budget of CONICYT has increased almost 2.7 times since 2008, from US\$182 million to US\$485 million in 2012. Projects in basic science supported by FONDECYT (a subdivision of CONICYT) have increased by 50% since 2010. No discipline in S&T has funded less than 40% of proposals (1). Postdoctoral fellowships in Chile and abroad increased by more than 40% in the past year (2), and they are expected to increase again after the next call [increases to FONDECYT's budget and Becas Chile are shown on p. 319 of the Budget of the Nation 2012 (3)]. Eleven recipients of the National Prize in Science, including the President of Academy of Science, applauded this change (4).

Contrary to the impression given by Astudillo *et al.*, CONICYT has indeed made research plans, which have been published in the media and discussed in forums with university authorities and scientists (5). A major share of CONICYT's budget is devoted to the scholarship program Becas Chile, an effort amounting to about US\$200 million in 2012,

allowing 3240 Ph.D. students to complete studies in Chile or abroad in 150 universities and programs around the world (4). Estimates show that by 2014, more than 800 Ph.D.s per year will graduate from national and international universities and join the ranks of scientists in Chile's universities, government, and private sector.

By the end of this year, CONICYT will implement a new fund (FONDEQUIP) aimed at increasing scientific competitiveness through access to modern laboratory equipment [(3), p. 320]. Moreover, all Chilean scientists and graduate students will have free electronic access to the major international journals at their universities. Recent data indicate that Chilean science exhibits the highest indicators among all Latin American countries in terms of papers published in journals with high ISI ratings per dollar invested and citations per paper (6). Fifty-three percent of ISI publications by Chilean scientists now have at least one foreign co-author (7).

Meanwhile, four international centers of excellence in the key areas of aquaculture biotechnology, communications and information research, mining, and pro-

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cessed foods have been launched in the past 2 years by INNOVA CORFO (the government innovation agency), attracting reputed institutions such as Fraunhofer, CSIRO, INRIA, and Wageningen University. By 2014, we expect that six more centers, which will receive a basal funding of up to US\$20 million in 10 years, will be ready to start operation.

A good example of the new times for science and technology is astronomy. By 2020, Chile will host almost 70% of the observation capacity in the world, involving a foreign investment of close to US\$5.8 billion (8). Chilean astronomers have rights to 10% of the observation time; thus, faculty at astronomy departments in Chilean universities have increased by 50% since 2007 and

more than 80 Ph.D. and Master students are enrolled in their programs (9).

Ministries of Science and Technology (MSTs) are not the only alternative for policy and promotion of science. In many instances, MSTs are synonymous with bureaucracy and prone to political favoritism. Administrative costs in CONICYT constitute 3.8% of the budget, all programs have scientific advisory committees, and the top officials remained in their posts after the change in government in 2010. More important, CONICYT serves more than 10,000 scientists and graduate students in Chile and abroad. As is well known, this President has met with government authorities and the Congress to reassess the structure and role of CONICYT within the existing National

Innovation System created in 2006 (10).

Chile is not at risk of research funding cuts or in desperate need of planning, as Astudillo *et al.* assert. Our efforts are now centered on increasing the number of scientists (presently around 4500) because empirical evidence (11) suggests that larger investments in R&D must be accompanied by minds and hands to accommodate the increase.

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CORRECTIONS AND CLARIFICATIONS

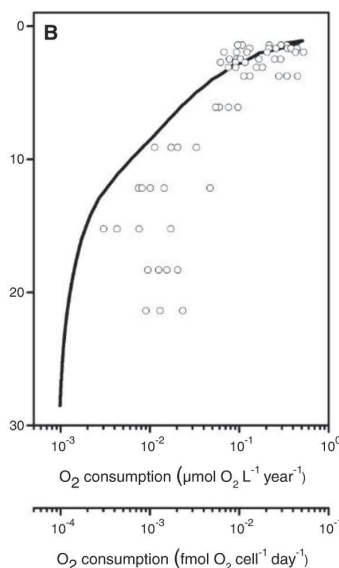
News Focus: "Near Eastern archaeology works to dig out of a crisis" by A. Lawler (18 May, p. 796). Henryk Witas is with the Medical University of Łódź, not the University of Łódź, in Poland.

Reports: "Observation of ^{239}Pu nuclear magnetic resonance" by H. Yasuoka *et al.* (18 May, p. 901). A minus sign was omitted from the Zeeman interaction expression in the first paragraph of the main text. The expression should be $H_z = -\mu_z \cdot B_z$.

Reports: "Aerobic microbial respiration in 86-million-year-old deep-sea red clay" by H. Røy *et al.* (18 May, p. 922). An older version of Fig. 2B was mistakenly published. The scale bar has been revised, and many of the data points in panel B have shifted accordingly. The correct version appears here.

Reports: "Extrachromosomal microDNAs and chromosomal microdeletions in normal tissues" by Y. Shibata *et al.* (6 April, p. 82). In reference 6, the first author's name was misprinted as Y. Hideo. The correct citation is: H. Yamagishi *et al.*, *Gene* **26**, 317 (1983). The correction has been made in the HTML version online.

Technical Comments: "Response to Comment on 'Fossilized nuclei and germination structures identify ediacaran 'animal embryos' as encysting protists'" by T. Hultgren *et al.* (9 March, p. 1169). The authors have posted an addendum to their Technical Response to address items in the final published Technical Comment that were not included in the version to which the authors responded. The addendum is posted here: www.sciencemag.org/content/335/6073/1169.4/suppl/DC1.



TECHNICAL COMMENT ABSTRACTS

Comment on "Seroevidence for H5N1 Influenza Infections in Humans: Meta-Analysis"

Maria D. Van Kerkhove, Steven Riley, Marc Lipsitch, Yi Guan, Arnold S. Monto, Robert G. Webster, Maria Zambon, Angus Nicoll, J. S. Malik Peiris, Neil M. Ferguson

A better understanding of the severity of H5N1 in humans is needed. Wang *et al.* (Brevia, 23 March 2012, p. 1463; published online 23 February 2012) overinterpret the results of seroprevalence studies and take too little account of underlying uncertainties. Although the true risk of death from H5N1 infection will likely be lower than the 60% of reported laboratory-confirmed cases, there is little evidence of millions of missed infections.

Full text at www.sciencemag.org/cgi/content/full/336/6088/1506-b

Response to Comment on "Seroevidence for H5N1 Influenza Infections in Humans: Meta-Analysis"

Taia T. Wang and Peter Palese

We address points made in the comment by Van Kerkhove *et al.* and explain why human H5N1 virus infections are much more common than was previously thought.

Full text at www.sciencemag.org/cgi/content/full/336/6088/1506-c

Letters to the Editor

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NEUROSCIENCE

The Eternal Silence of Neuronal Spaces

Stanislas Dehaene

Consciousness is, quite literally, mind-boggling. From the objective, third-person perspective of neuroscience, the subject consists of clarifying how three pounds of brain flesh, a mere assembly of molecules, can ever give rise to conscious mental states, feelings, and a sense of self. But every neuroscientist is also a human being, a self with a point of view, who suffers life's miseries and can therefore ponder the mysteries of conscious and unconscious thoughts from a privileged first-person perspective. Whence this unbearable toothache? Why the nostalgia of a lost love? Who is this I, really? Do "I" own my life, or do my genes, my brain, and my habits own me? And if so, what gives my life a meaning?

In *Consciousness*, Christof Koch, a cognitive scientist at Caltech, artfully weaves the two perspectives together. The resulting atypical science book combines a review of top-notch scientific findings with personal memories, musings, and confessions of an active, introspective, and perplexed neuroscientist.

The science itself is generally impeccable. As he has previously demonstrated (1), Koch excels at explaining, in simple and concise terms, the most recent research into the neurobiology of consciousness. Enlightening discoveries abound. With collaborators Itzhak Fried and Rodrigo Quiroga, the author initiated a remarkable search for the neuronal correlates of elementary conscious percepts, capitalizing on the novel capacity to record from individual neurons in epilepsy patients. They discovered that some neurons in medial temporal cortex fire in response to the face or name of a specific person. Critically, even for a constant stimulus, the neurons fire only when the subject reports seeing the picture and not when eclipsed by binocular rivalry or masking (2). Such neuronal observations converge nicely with brain-imaging experiments in normal volunteers to suggest that synchronous and distributed brain activity in specific higher-order brain

areas provides neuronal signatures of conscious brain states (3).

The operational knowledge of consciousness has reached the clinic. In a patient thought to be in a vegetative state, functional magnetic resonance imaging detected complex mental states that reflect a conscious mind (4), and simpler detection devices are now being envisaged. Pursuing consciousness at the cellular and molecular levels, Koch has launched a \$300-million program at the Allen Institute in Seattle to develop "brain observatories" that will dissect the microcircuitry of visual cortices in mice (5).

Koch fearlessly discusses some of the most difficult questions in the field. For instance, are animals conscious? Koch's guess is a resounding yes: his six dogs surely have conscious states—their tails, snouts, paws, bod-

Consciousness Confessions of a Romantic Reductionist

by Christof Koch

MIT Press, Cambridge, MA,
2012. 193 pp. \$24.95, £17.95.
ISBN 9780262017497.

BROWSINGS

Atlas of Human Infectious Diseases. Heiman F. L. Wertheim, Peter Horby, and John P. Woodall, Eds. Wiley-Blackwell, Oxford. 2012. 308 pp. £84.99, \$130, €102. ISBN 9781405184403.

If you're interested in infectious diseases and you like maps, you'll love leafing through the *Atlas of Human Infectious Diseases*. It shows the global distribution of more than 110 diseases, from well-known scourges such as malaria and cholera to oddballs like strongyloidiasis and O'nyong'nyong virus disease. Maps have been available for many diseases, but, Wertheim notes, it's often unclear who made them or what data were used—and they frequently contain errors.

Wertheim (a clinical microbiologist working in Hanoi for the Wellcome Trust and Oxford University) didn't want an electronic atlas but a book you can draw inspiration from as you read it on the couch. The editors took five years to prepare the maps, sifting through data from countless papers, field reports, and other sources. Each map was reviewed by two experts for its particular disease. The atlas also charts underlying factors such as water and sanitation, international travel, and urbanization.

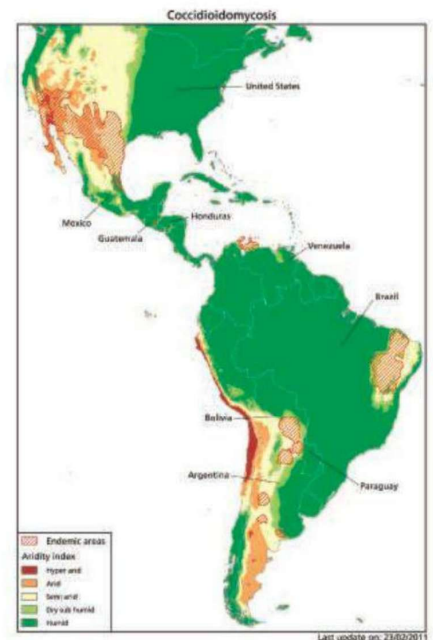
Infectious diseases are nothing if not dynamic, and freely accessible updates will appear on a forthcoming companion website. Meanwhile, Wertheim hopes that the gaps in the maps will inspire researchers to collect more data on where pathogens occur. For many diseases, Africa is epidemiology's terra incognita—as painfully large gray areas in the atlas testify.

—Martin Enserink

ies, ears, and tongues obviously express a cornucopia of internal feelings. "Indeed," he whimsically remarks, "I often think dogs are closer to true Buddha nature than people are." Here, alas, he offers no experimental data; avowedly, only the dog owner speaks, not the scientist and even less the dog.

From dog to god is a small step. Perhaps the author's most unexpected confession is that he was a long-time devout Christian. Although those days are gone, Koch still feels strongly that life must have a purpose. In the introductory chapter, he confesses, "With perfect hindsight, I now realize that what drew me to studying consciousness was a compelling and entirely subterranean desire to justify my instinctual belief that life is meaningful." Perhaps this stance explains his strong attraction to the philosopher David Chalmers's dual-aspect theory and, especially, to Giulio Tononi's mathematical theory of consciousness as integrated information (6), which he describes as amounting to "a form of property dualism." In a curious move, our romantic reductionist now concludes that the mental and the physical compose "two sorts of properties ... that can't be reduced to each other."

The implications of Tononi's theory fill Koch with uncritical enthusiasm. He expresses his strong faith that the theory



Coccidioidomycosis (valley fever).

will soon lead to the clinical application of a consciousness-o-meter for coma patients, although Tononi's measure of consciousness Φ remains essentially noncomputable. Building on the theory, the author offers a grandiose panpsychic vision, which he finds "terribly appealing for its elegance, simplicity, and logical coherence." Every organism, even bacteria, would possess some degree of consciousness. The logical coherence escaped me: Only two chapters earlier, Koch was showing, there with strong empirical data, how complex networks of the human cerebellum and even the cortex operate entirely unconsciously. During the attentional blink, for instance, word meanings can be accessed nonconsciously, a feat that necessarily involves differentiated and integrated networks of the left temporal lobe. Tononi's theory makes no contact with such observations yet.

"The theory," Koch writes, "has profound consequences that bear some resemblance to the prophetic ideas of Pierre Teilhard de Chardin." Evolution implies a progressive amplification of global consciousness, with computers and the Internet as its most recent avatars. Openly confessing his recent familial turmoil and his loss of Christian faith, Koch finds solace in this view of life. He also admits that his long-time mentor and collaborator on the mind-body problem, Francis Crick, would have cringed. An avid reader of Jacques Monod's *Chance and Necessity* (7), I too was stunned: Rare are contemporary biologists who confess such thoughts, and they are even rarer, I thought, among those who study consciousness.

With its fascinating glimpses into the budding science of consciousness and the intermixed frank autobiographical notes (qualms of conscience included), *Consciousness* offers a charming read. Behind the lines, however, lies a darker message: neuroscience obviously cuts deep into one's life. One is inescapably reminded of a famous psychology experiment (8) in which reading a passage of Francis Crick's *Astonishing Hypothesis* (9)

on the genetic and neuronal determinants of behavior (thus weakening beliefs in free will and responsibility) increased cheating behavior. Can neuroscience be reconciled with living a happy, meaningful, moral, and yet nondelusional life? I will confess that this question also occasionally keeps me lying awake at night. However, I cannot but think that Koch is, unfortunately, misguided and that Crick and Monod are, unfortunately,

right: In the eternal silence of neuronal spaces, our anguish probably goes unheeded. My own optimism consists of enjoying every single moment of consciousness while it lasts.

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10.1126/science.1222480

PSYCHOLOGY

Facilitating "A-ha!" Moments

Anna Vlasits

Creativity is an ill-defined skill that almost everybody wants. How can we attain it? Although I don't have the answer to this question, reading Jonah Lehrer's *Imagine* might lead one to some possibilities.

Full of suggestions and anecdotes, the book reads like a creativity self-help guide pumped up with the latest psychology and business research. In one chapter, Lehrer (a

science writer) recounts how Scotch tape was invented. In another, he describes how psychologists discovered that uncritical brainstorming reduces creative output. Lehrer emphasizes that there are actually many different kinds of creativity, including right-brain creativity (implied in "a-ha!" moments of

insight), left-frontal-cortex creativity (controlling highly focused, sustained creative output), and "innovative" creativity (as in cutting-edge businesses such as 3M, Pixar, and Google as well as groups of artists making Broadway musicals).

Although the term "creativity" remains ill-defined throughout the book, by cutting the topic up into little pieces Lehrer hopes to get to the bottom of how humans become creative. *Imagine* is addictive, probably because the book contains information about our behavior that all of us have observed. I too work in coffee shops to get my imagination flowing; have insights while taking showers; and live in a city, where creative output is at its highest. Even more enticing are all of the little tricks that Lehrer pulls from psychology research on creativity. For instance, putting bathrooms in a space that forces people with different expertise to interact increases a company's creative output. Lehrer argues that anyone can be creative. It doesn't matter how drab you're feeling today or whether you have skills or training. Surround yourself with environmental conditions conducive to creativity, and you can produce something great.

The book aims to stimulate people to be as creative as possible. In fact, Lehrer holds that we should change our education system to emphasize teaching children to be creative. This raises the question of what we really get out of being more creative as a community. The two main outputs of creativity that Lehrer describes are technological innovations (read patents and therefore money) and art (read entertainment). It appears that he thinks creativity results in making either something that can be sold to people or something that will entertain them—or both.

There seems something sinister about reducing creativity to a checklist of to-dos and reducing the goals of our creative output to wealth and art. Whereas Lehrer acknowledges that "the creative process will never be easy, no matter how much we know about neurons and cities and Shakespeare," he does not address the important issue of what we should do with our creativity. Imagination can be used to invent a new way to build better solar panels or to cheat people out of money. Describing ways to direct human creativity toward good ends would certainly constitute a creative book.

That said, Lehrer smoothly and engagingly blends scientific findings with stories about creative breakthroughs. *Imagine* is just plain fun to read, and the author's neat prose dishes out valuable information. While writing this review, I couldn't help but match my creativity-related behaviors to his descriptions, and I consciously referred to the book for tips to make the process easier. I don't know whether doing so improved the results, but it certainly got my imagination going.

10.1126/science.1224275

Imagine

How Creativity Works

by Jonah Lehrer

Houghton Mifflin Harcourt,
Boston, 2012. 299 pp. \$26.

ISBN 9780547386072.
Canongate, London. £18.99.
ISBN 9781847677860.

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SCIENCE EDUCATION

Engaging Teachers, Scientists, and Multimedia to Promote Learning

Dennis Liu,* Satoshi Amagai, Jennifer Bricken

There are many Web sites supporting instruction in the life sciences (1). One such effort, the Howard Hughes Medical Institute's (HHMI's) BioInteractive (biointeractive.org), developed from a focus on practicing scientists explaining their research; engaging explanations driven by compelling examples and graphics; and meaningful dialogue with instructors to improve products and to facilitate classroom adoption of materials (2).

BioInteractive is a library of multimedia resources to support teaching. The content is a product of interactions between scientists and educators, mediated by a team of production specialists. To make materials convenient for use in teaching, content is free and offered in a variety of formats, for example, video streamed or for file download. Among those who voluntarily provide feedback, the majority identify themselves as high school and college educators, although students also visit the site. For notices of new programs, 24,000 educators are currently registered. Over 1 million unique visitors come to the site annually, exploring, on average, eight features. The average viewing time of a visitor streaming a lecture video is >30 min.

BioInteractive arose to provide online support to the HHMI Holiday Lectures on Science, presented by leading scientists and including videos, scientific demonstrations, and animations. Advances in online video make the lectures a resource for teachers and students.

The Web site includes lectures, interactive features, and short films (see the image). Many videos feature scientists at different career stages, from undergraduates to senior research professors, talking about their research and their lives as scientists. The site also has teacher guides, lesson plans, instructions for hands-on activities, and virtual labs. BioInteractive also hosts >120 animations. Engaging animations can be instructional tools to show the sequence of complex events, to compress time and space, and to reveal otherwise invisible structures and processes



Image from BioInteractive, based on the work of B. Bassler and colleagues on bacterial quorum sensing.

(3–6). They can encapsulate research findings from dozens of technical publications.

The BioInteractive development team works closely with teachers to develop interactive features, teacher guides, standards and curricula correlations, lessons, and hands-on activities that make use of and extend the multimedia resources. We look for consensus among teachers over whether materials meet various local and national standards and are likely to fit the curricula of classroom teachers. We have adapted our materials to the Advanced Placement (AP) biology curriculum and have published guides tying BioInteractive materials to topics in the high school curriculum, such as biotechnology or immunology. Efforts are under way to correlate content with the new AP biology framework and the Next Generation Science Standards.

Teachers are contacted online via surveys and forums but mostly through workshops. Each year, ~14 teachers attend the Holiday Lectures and work for an additional 3 days on ideas for using the lectures in the high school classroom. This workshop develops support materials for the current series, as well as ideas for future lecture series and short films. For a larger network of teachers, the BioInteractive team organizes additional workshops nationwide. In 2011, HHMI had direct contact with an estimated 3500 teachers in 41 workshops and sent materials to another 31 professional development events.

We are exploring ways to grant continuing education credits to teachers who participate in our workshops and also for those seeking credit for using our online materials. Despite variability (each state and district has a specific set of criteria for licensing and credentialing teachers for promotion), there are many commonalities, and virtually all science

BioInteractive focuses on scientists and their research, while engaging with teachers to improve educational materials and practice.

teachers participate in ongoing professional development (7).

As the audience has grown, BioInteractive has enjoyed favorable recognition (8). Teacher enthusiasm for the materials is high, but we have not yet directly evaluated student enthusiasm or measured learning outcomes. To address this, we have begun pilot projects to field-test methods for directly measuring the impact of various Web features on student learning in a classroom setting, as well as in a setting without instructors. We hope to learn whether instructor enthusiasm for the materials is well founded, as well as to understand the impact that educational videos can have when used by students as a component of free-choice learning (9).

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Acknowledgments: The authors thank the HHMI Educational Resources Group: O. Ajagbe, L. Bonetta, E. Clements, E. Keller, T. Reid, B. Pietch, B. Porch, D. Thomas, D. Trackman, and C. Vargas. Special thanks to P. Bruns, vice president emeritus, HHMI Department of Grants and Special Programs, and to S. B. Carroll, vice president, HHMI Science Education.

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10.1126/science.1220252

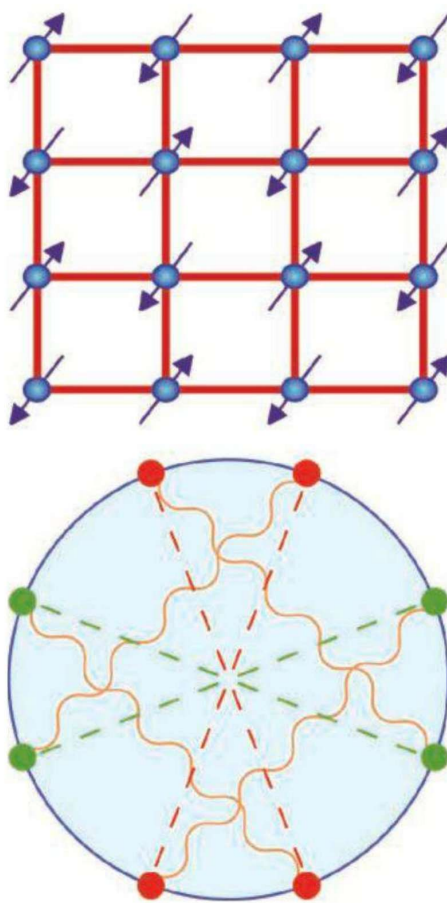
PHYSICS

Entangling Superconductivity and Antiferromagnetism

Subir Sachdev

Today we have two families of high-transition temperature (T_c) superconductors, based respectively on compounds in which copper and iron atoms occupy a layered square lattice. An open question is how the quantum mechanics of electrons moving cooperatively on such lattices leads to high- T_c superconductivity. Both families display antiferromagnetism as their chemical compositions are varied (see the figure). It is the interplay between the magnetic and electronic properties that is thought to be controlled by intricate quantum entanglement among the electrons, and to be at the origin of the superconducting properties. The antiferromagnetism is strongest at compositions at which T_c is either zero or small. As the composition is varied and the antiferromagnetism decreases, a critical composition is reached at which the antiferromagnetism vanishes at zero temperature—an example of a quantum phase transition. On page 1554 of this issue, Hashimoto *et al.* (1) report observations of an especially well-characterized example of such a quantum critical point in a high- T_c superconductor, crystals of $\text{BaFe}_2(\text{As}_{1-x}\text{P}_x)_2$ with minimal chemical disorder. A novel feature of their experiments is that the signature of a magnetic critical point is observed in an electrical property: The antiferromagnetic quantum critical point leads to a change in the ability of the electrons to carry a supercurrent. The results demonstrate the close connection between antiferromagnetism and high- T_c superconductivity.

Low-temperature superconductors such as mercury are understood by the 1957 theory of Bardeen, Cooper, and Schrieffer (BCS). A key feature of the theory is that pairs of electrons bind to form particles known as Cooper pairs, which are bosons. These bosons can then undergo condensation into a common quantum state, and this explains much of the phenomenology of the traditional superconductors. The pair binding of the electrons requires an attractive potential between them, and this appears when the electrons exchange quanta of lattice vibrations—phonons.



Extending this BCS picture to the high- T_c superconductors requires a stronger attractive potential, stronger than the lattice vibrations can provide. One possible source is the antiferromagnetism where the electrons can exchange quanta of the “vibrations” of the local antiferromagnetic order, which is linked to fluctuations of the electronic spin. Provided the coupling constant of this exchange process is small, a reliable theory of superconductivity can be developed using the BCS framework. One of the predictions of such a theory (2) is that the Cooper pairs that form via this mechanism must have a wave function that changes sign when the momenta of their constituent electrons are moved through the range of possible values. In the copper-based superconductors, such Cooper pairs have d-wave symmetry (see the figure). Such a sign change has

Common features found in two families of materials may help explain the mechanism of high-temperature superconductivity.

Find a partner. (Top) Antiferromagnetism on the square lattice of Cu ions in a high- T_c superconductor. The arrows indicate the orientation of the electron spins. In a ferromagnet all electron spins are parallel, whereas in an antiferromagnet they form a checkerboard pattern. (Bottom) A picture of the occupied electron states in the momentum space of a metal; its boundary is the Fermi surface. Eight particular single-electron states on the Fermi surface are indicated by the small circles. The wavy lines connect electrons that can scatter into each other via exchange of a quantum of an antiferromagnetic spin fluctuation. The dashed lines connect electrons that form Cooper pairs. The Cooper pairs of the red circles have a wave function with the opposite sign from the green circles, a characteristic feature of superconductivity mediated by antiferromagnetism. Note that the wavy lines only connect circles with different colors.

been observed in both classes of superconductors (3, 4). However, BCS theory cannot explain high- T_c superconductivity because it is only valid when the coupling constant is small. We cannot simply assume that larger coupling constants will lead to higher T_c values, because increasing the coupling constants leads to several new effects that are not included in the BCS theory, some of which are detrimental to superconductivity.

One method of increasing the coupling strength is to approach the antiferromagnetic quantum critical point (5). Here the attraction does increase, and, moreover, beyond-BCS effects can be systematically studied. The stronger coupling leads to strong scattering in which the electrons lose most of their energy to the quanta of the collective antiferromagnetic fluctuations, the electron-like particles of the metal become heavier, and some of them lose their integrity (6); this is detrimental to superconductivity because it is these very particles that are the constituents of Cooper pairs. Should some of the electrons form Cooper pairs anyway, the resulting modification of the Fermi surface of the metal (see the figure) can suppress antiferromagnetic fluctuations needed for the pairing of the remaining electrons. And finally, other types of ordering can appear as by-products of the stronger coupling, such as the formation of stripes. Recent work (7) has argued that the Cooper pair formation nevertheless remains the dominant consequence of the

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strong coupling of the electrons to antiferromagnetic spin fluctuations at the critical point, and that high- T_c superconductivity is the most likely consequence.

Hashimoto *et al.* show a clear new signature of this tug-of-war between antiferromagnetism and superconductivity. T_c is at a maximum close to the antiferromagnetic quantum critical point, signaling that antiferromagnetic quantum critical fluctuations do indeed enhance Cooper pair formation. On the other hand, their measurements of the extent to which a magnetic field can penetrate the superconductor at zero temperature show, surprisingly, that this length is also a maximum at the quantum critical

point. A large penetration depth implies that the ability of the electrons to carry a supercurrent is actually at a minimum at the quantum critical point. One possible explanation is that the electrons, and so the Cooper pairs, have an average effective mass that is larger at the critical point, and this impedes their motion. Such an enhancement in the mass of the electrons is a natural consequence of the strong scattering by the antiferromagnetic spin fluctuations. Thus, the maximum in T_c —and the concomitant maximum in the penetration depth—constitute evidence for the opposing tendencies in the influence of the antiferromagnetic quantum critical point on high- T_c superconductivity. These

observations will be valuable in the ongoing theoretical effort to unravel the quantum interplay between antiferromagnetism and superconductivity.

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10.1126/science.1223586

CELL BIOLOGY

A Unifying Role for Prions in Neurodegenerative Diseases

Stanley B. Prusiner

Many neurodegenerative diseases—including Creutzfeldt-Jakob disease, Alzheimer's disease (AD), Parkinson's disease, and amyotrophic lateral sclerosis (ALS)—share two remarkable characteristics. The first is that more than 80% of cases are sporadic. The second is that although many of the disease-specific mutant proteins are expressed in embryogenesis, the inherited forms of these neurodegenerative diseases are late-onset. This suggests that some event occurs with aging that renders a disease-specific protein pathogenic. More than 20 years ago, I argued that this event involves a stochastic refolding of the etiologic protein into a misfolded infectious state known as a prion. In the past decade, there has been renewed interest in the possibility that the proteins causing neurodegeneration are all prions, which would profoundly influence the development of diagnostics and effective therapies.

Many diverse explanations for the late onset of neurodegenerative diseases have been offered, including oxidative modifications of DNA, lipids, and/or proteins; somatic mutations; modified innate immunity; exogenous toxins; RNA-DNA differences; chaperone malfunction; and haploinsufficiency. An alternative unifying explanation is that a

diverse group of proteins can form prions. Although small numbers of prions could be cleared by protein degradation pathways, accumulation above a certain threshold over time would enable the prions to self-propagate (see the figure), resulting in central nervous system (CNS) dysfunction (1).

Fungal prions have been invaluable in defining the spectrum of prions. Although yeast prions are not infectious in the sense of being released into the culture medium and infecting other yeast, they are transmissible from mother to daughter cells and thus can readily multiply. Interestingly, many of the mutant proteins causing heritable neurodegenerative diseases are found in insoluble disease-specific aggregates known as amyloid deposits, such as plaques, neurofibrillary tangles (NFTs), and Lewy bodies (see the figure and table S1). Similarly, most fungal prions have a high β -sheet content and can polymerize into amyloid fibrils. That said, it is important to distinguish between prions and amyloids: Prions need not polymerize into amyloid fibrils and can undergo self-propagation as oligomers. The self-propagation of alternative conformations is a key feature of all prions.

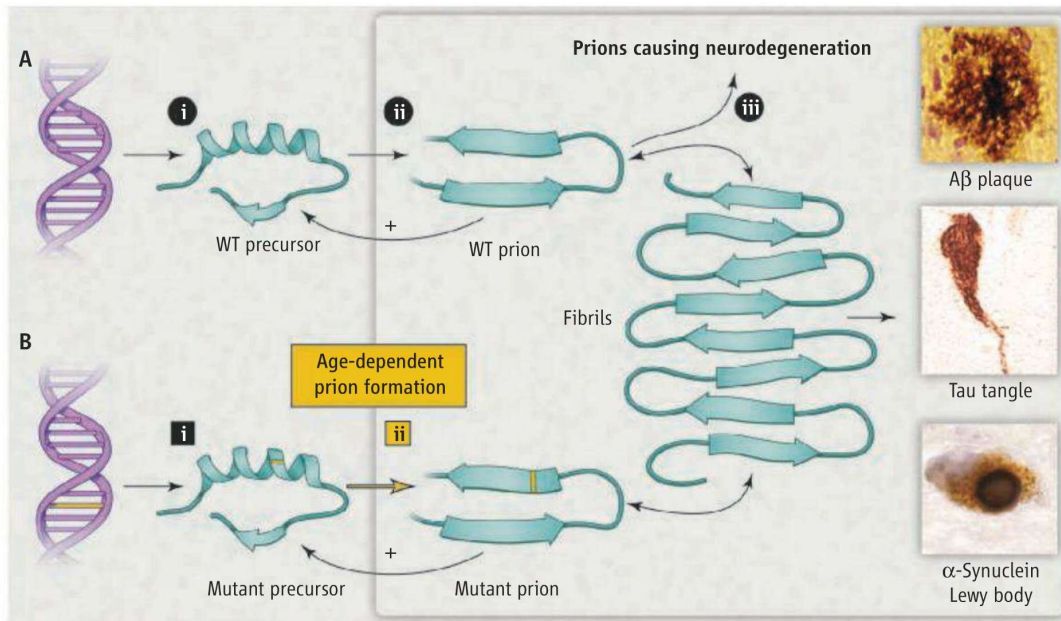
Substantial experimental evidence has now accumulated to support a unifying role for prions in neurodegenerative diseases. In AD, for example, which is characterized by the deposition of A β amyloid plaques (see the figure), Ridley and Baker performed a set of

A profound change in thinking about the etiologies of many neurodegenerative diseases has far-reaching implications for developing therapeutics.

heroic experiments in which they inoculated human AD brain homogenates intracerebrally into marmosets. The marmosets developed A β amyloid plaques with incubation periods exceeding 3.5 years (2), demonstrating for the first time that the disease is transmissible and thus supporting the existence of a disease-causing prion. Similar results have been shown by Walker and Jucker and others using transgenic AD mice (3, 4). Importantly, the disease agent has been identified as consisting solely of A β prions using synthetic A β peptides (5).

The tauopathies are a group of neurodegenerative diseases characterized by tau protein aggregation. Mutant tau has also been shown to be transmissible using transgenic mice (6), with tau aggregates being observed 1 year after inoculation. In addition, an aggregated segment of the tau protein initiated tau prion formation after being introduced into cultured cells (7). Among the tauopathies, the frontotemporal dementias (FTDs) are particularly interesting because they sit at the interface between psychiatry and neurology. Often, psychiatrists see FTD patients for years before recognizing subtle but progressive deterioration and referring them to neurologists. Aggregates of tau prions in the frontal lobes can produce inappropriate social interactions, depression, and diminished executive function as well as insomnia; later, drug abuse, alcoholism, and suicide may occur. The discovery that some contact-sport athletes, as

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Modeling neurodegeneration caused by prions. (A) Wild-type (WT) prions multiply through self-propagating cycles of post-translational modification; generally, an increase in β -sheet content accompanies prion formation. Pathogenic prions are most toxic as oligomers and less toxic after polymerization into amyloid fibrils. Depending on the protein, the fibrils coalesce into amyloid plaques, neurofibrillary tangles, or intracellular inclusions such as Lewy or Pick bodies. Drug targets for the development of therapeutics (black circles): (i) lowering precursor protein, (ii) inhibiting prion formation, and (iii) enhancing prion clearance. (B) Late-onset heritable neurodegeneration argues for two discrete events (squares): (i) mutant protein synthesis and (ii) prion formation.

well as soldiers from the Iraq and Afghanistan wars, develop posttraumatic FTDs with psychiatric symptoms—often called post-traumatic stress disorders or PTSD initially—has begun to clarify how diverse neurological insults can all produce NFTs composed of tau prions (8, 9). Some of the variations in the clinical presentations of the tauopathies may be due to different prion strains, which represent distinct conformations (10).

Classical scrapie prions in ovines and rodents have been shown to spread throughout the peripheral nervous system and CNS. Consistent with the concept that other neurodegenerative disease-causing proteins are also prions, Heiko Braak and colleagues demonstrated the spreading of A β amyloid plaques and NFTs in AD from the entorhinal cortex to many regions of the cerebrum (11). Presumably, the A β prions spread through the extracellular space, whereas tau prions seem more likely to move between neurons trans-synaptically (12). Recent studies have traced the spread of tau prions using functional magnetic resonance imaging intrinsic connectivity analysis in several tauopathies (13).

Parkinson's disease is characterized by the accumulation of α -synuclein into so-called Lewy bodies in neurons. The finding of Lewy bodies in grafted fetal brain cells a decade after transplantation into Parkinson's patients raised the possibility that α -synuclein proteins

can also become prions that were synthesized in these grafted cells (14). The surface of Lewy bodies is covered with fibrils composed of β sheet-rich α -synuclein proteins (see the figure). The normal form of α -synuclein seems to be either unstructured or high in α -helical structure, but like other prion proteins, α -synuclein can adopt a β sheet-rich conformation. Although unproven, it seems likely that β sheet-rich α -synuclein prions crossed from the transplanted patient's own neurons into the grafted cells and induced a change in the structure of α -synuclein (15). Once established, this process became self-propagating, as with all pathogenic prions. Further evidence for α -synuclein prions comes from studies with recombinant α -synuclein assembled into fibrils that induced α -synuclein prions to multiply in both cultured cells and transgenic mice (16, 17).

Increasing evidence argues that prions cause some forms of ALS and may feature in the pathogenesis of Huntington's disease. More than 60 different mutations in superoxide dismutase (SOD1) have been found to cause familial ALS. Aggregates of mutant human SOD1 have been shown to be self-propagating in cultured cells and, as such, are prions (18, 19). Studies of expanded polyglutamine repeats in a huntingtin protein fragment demonstrated self-propagation of spontaneous aggregates in cultured cells; that is,

they are prions (20). Huntingtin prions explain why people with 5 to 10 additional glutamines do not become ill until they are 40 to 60 years of age even though the mutant protein is synthesized in embryogenesis.

Not all animal prions cause disease. Some mammalian prions, such as cytoplasmic polyadenylation element-binding (CPEB) protein, mitochondrial antiviral-signaling protein (MAVS), and T cell-restricted intracellular antigen 1 (TIA-1), perform important cellular functions (21–23), including regulating gene transcription and the immune response. Unexpectedly, the biologically active forms of CPEB and MAVS are the oligomeric prion states and not the monomeric precursor proteins.

The convergence of studies demonstrating prions in the pathogenesis of common neurodegenerative maladies has been remarkable (table S1). Many mysteries are now explicable within the framework of the prion concept. Most important, strategies for developing informative molecular diagnostics and effective therapeutics for these elusive disorders emerge from our knowledge of prions (see the figure). Early diagnosis will require reporters such as positron emission tomography ligands to identify prions long before symptoms appear. Meaningful treatments are likely to require cocktails of drugs that diminish the precursor protein, interfere with the conversion of precursors into prions, and/or enhance the clearance of prions.

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Supplementary Materials

www.sciencemag.org/cgi/content/full/336/6088/1511/DC1
Table S1

10.1126/science.1222951

MOLECULAR BIOLOGY

Genetic Events That Shape the Cancer Epigenome

Russell J. H. Ryan^{1,2} and Bradley E. Bernstein^{1,2}

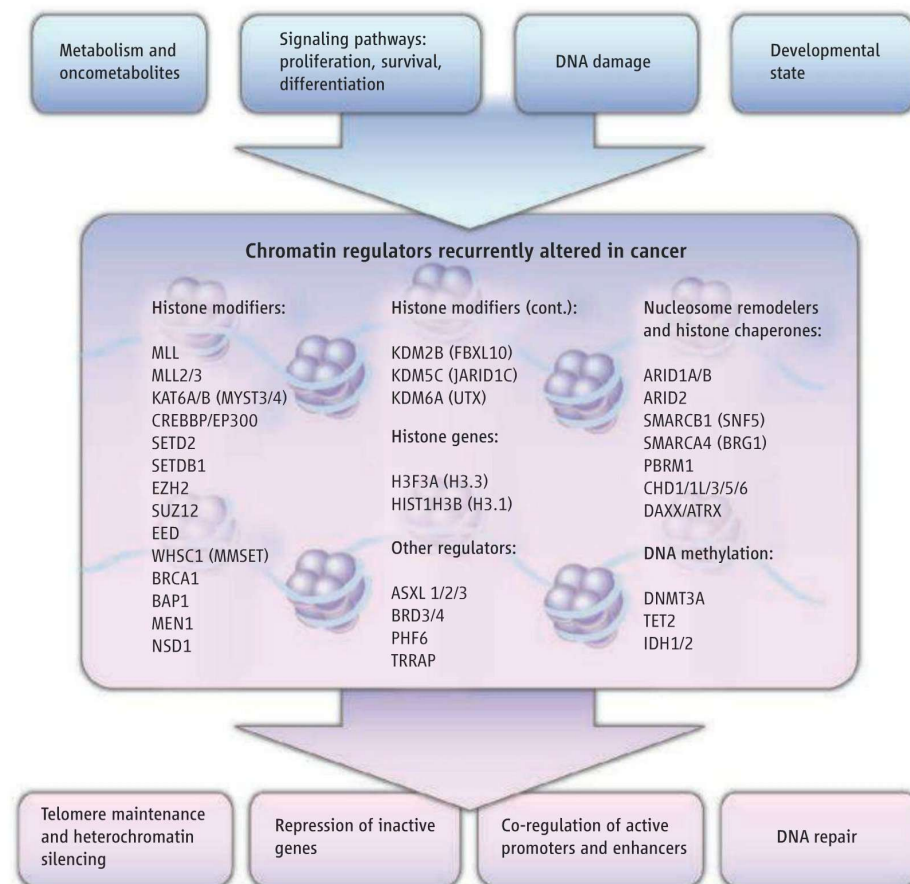
Mutations in chromatin-related genes in human tumors support a role for epigenetic mechanisms in driving cancer.

Since the discovery of the first recurrent mutations in oncogenes and tumor suppressor genes, it has been clear that cancer is, in large part, a genetic disease. Yet nearly every human neoplasm retains a phenotype reflective of its tissue of origin, thus underscoring the centrality of epigenetics in cancer biology. Indeed, there is increasing recognition that transmissible epigenetic changes—chemical modifications to the genome or its scaffold that do not involve a change in the nucleotide sequence—may be acquired *de novo*, and that these “epimutations” may also contribute to carcinogenesis. Aberrations of DNA methylation have epitomized this concept, largely because of the direct mechanism by which hypermethylation of a DNA locus can be faithfully transmitted through cell division. Localized hypermethylation of silenced gene promoters and global DNA hypomethylation are characteristic features of many human tumors (1, 2). However, the idea that histone modifications and other chromatin features also mediate epimutations in tumors has been more controversial, in part due to the obscurity of models for direct epigenetic transmission (3). The recent flurry of reported mutations in chromatin-related genes in human tumors indicates the need to reassess the perceived roles for chromatin and epigenetic mechanisms in cancer biology.

Histones and associated chromatin proteins control the accessibility of genes and

genomic elements and thereby influence their targeting by protein machinery. Regulatory elements in the genome are exposed when chromatin is in a permissive confor-

mation that is conducive to transcription factor binding, whereas inactive loci, regions of repeated DNA sequence, and telomeres (repeated sequences at the ends of chromosomes) are organized into more compact structures. Much progress has been made in understanding how histone-modifying enzymes and other chromatin regulators (CRs) collaborate with transcription factors to translate cell signaling inputs into appropriate transcriptional outputs, playing roles in initiation, elongation, splicing, and repression. Chromatin also affects global genome architecture in a dynamic fashion. Although the epigenome—the totality of chemical changes to DNA and histones—is plastic in embryonic cells, large swaths of DNA become sequestered within compact heterochromatin and nuclear lamina-associated domains during cell differentiation and commitment (4). These structures can be further modulated by cellular events, such as epithelial-mesenchymal transition, metabolic changes, and aging (2, 5, 6). For example, the oncometabolite 2-hydroxyglutarate, generated by mutant isocitrate



Chromatin, mutations, and cancer. Cancer genome studies have uncovered recurrent mutations in numerous chromatin regulatory genes. An important goal will be to understand how the resulting chromatin alterations affect transcriptional regulation, genome stability, telomere maintenance, and other aspects of cell physiology, and to determine which of these effects drive cancer fitness.

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dehydrogenase 1 and 2, can globally reprogram the methylation of DNA cytosine and histone H3 Lys⁹ by inhibiting TET family DNA hydroxylases (7) and Jmjc family histone demethylases (8), respectively.

CRs also play central roles in genome replication, integrity, and repair (9), thus suggesting mechanisms by which alterations of chromatin machinery may promote genomic aberrations. Certain regulators are critical for telomere maintenance and cellular life span; others act in pericentromeric, telomeric, and subtelomeric heterochromatin to maintain the stability and transcriptional silencing of repetitive DNA. These processes are frequently altered in cancer (9–11).

Critical events in the regulation of chromatin state involve the addition, removal, or binding of histone modifications, remodeling or exchange of nucleosomes (the fundamental packing unit of eukaryotic chromatin), and alteration of high-order chromosomal organization. Remarkably, cancer genome sequencing studies have unveiled an abundance of somatic mutations in genes encoding CRs that are responsible for each of these biochemical activities (see the figure). Even histone genes themselves can be mutational targets, as seen in the recent discovery of recurrent mutations in histone H3 genes (along with histone H3.3 chaperones) in pediatric glial cancers (12, 13). Specific CR mutations appear to be the dominant genetic event in certain pediatric tumors that show few additional genetic aberrations, such as infantile rhabdoid tumors (SMARCB1 inactivation) (14), demonstrating their substantial oncogenic potency when juxtaposed upon an epigenetically permissive developmental state. The frequent occurrence of CR mutations across many cancer types blurs the distinction between cancer genetics and epigenetics, as oncogenic chromatin alterations that might not be epigenetically stable on their own could be maintained by genetic mutations.

Identifying the mechanisms by which CR mutations contribute to tumor fitness has thus become a major challenge in cancer research. Many CRs bind to thousands of genomic loci and influence (directly or indirectly) the expression of multiple genes and pathways. Within any given cellular context, alteration of a specific CR may yield functionally important changes only at a subset of its binding sites, and only a fraction of these may be important in disease initiation or maintenance (15). Identifying the subset of physical and regulatory interactions relevant to a malignant phenotype will require a combination of

genetic modeling of CR mutations with multifaceted experimental readouts of chromatin, transcriptional, and phenotypic states.

The proliferation of genome-scale technologies for studying chromatin structure presents many options for investigating oncogenic CR mutations. Such approaches have already enabled the annotation of large numbers of enhancer-like sequences in the human genome, as well as other types of “functional elements” whose activities vary among cell types (4, 16). They have also revealed a division of labor among CRs, with specific CR complexes, or combinations thereof, partitioning among different classes of elements (17). Many of the CRs that are recurrently altered in cancer, such as SWI/SNF chromatin remodelers and the acetyltransferases CBP and p300, bind and potentially regulate enhancers near genes that function in differentiation, cell signaling, and other context-specific pathways that may thus be influenced by the mutational events.

Cancer mutations have also surfaced in CRs that mediate gene silencing, including enzymes that catalyze repressive histone modifications or remove activating marks. Homologs of the *Drosophila melanogaster* Polycomb genes have drawn particular interest, as they have been linked to cancer stem cell proliferation and represent promising therapeutic targets. Mammalian Polycomb proteins interact in combinatorial fashion to form multiple complexes that silence different types of genomic loci in different cellular contexts (18). These include classical regulators that interact with methylated histone H3 Lys²⁷, as well as complexes linked to less familiar modes of heterochromatin silencing.

Combinatorial CR binding is also evident at promoters, where occupancy patterns distinguish sets of genes with coherent functions related to controlling the cell division cycle, stress response, and other processes (17). These patterns likely relate to distinct regulatory modes at play in the different promoter sets, such as transcriptional initiation or elongation, pausing of RNA polymerase, and stable or reversible chromatin repression. Notably, global mapping studies suggest that critical transcriptional regulatory modes are altered by MLL fusion proteins in some leukemias (15). Considerable efforts will be needed to understand how CR mutations alter chromatin patterns within promoters and other elements, and whether chemical inhibitors of chromatin enzymes can play a role in correcting such defects.

Cancer types in which multiple CRs are mutated present further conceptual chal-

lenges. For example, myeloid neoplasms show recurrent and often co-occurring mutations in numerous genes affecting both Polycomb activity and DNA methylation, requiring that mechanistic models account for how these aberrations interrelate in neoplastic initiation and progression (7). Moreover, the concept that a single CR can play divergent roles in different malignancies is raised by the mutational profile of the human gene encoding the Polycomb enzyme EZH2, which is a frequently inactivated tumor suppressor in myelodysplastic syndromes (19) yet shows enzymatic gain-of-function mutations in germinal center B cell lymphomas (20).

A central goal of investigating cancer-related CR aberrations is to develop new therapeutic approaches. Chromatin alterations driven by CR mutations might be reversed by direct pharmacological targeting of oncogenically activated CRs, or by modulating other CRs to “correct” aberrant chromatin states. Identifying and targeting downstream “driver pathways” that mediate the oncogenic effects of CR mutations, such as apoptotic or cell cycle pathways, may also prove efficacious. The diverse effects of CR mutations may also provide opportunities for synthetic lethal strategies, as a CR mutation selected for its oncogenic effect on one pathway may remodel other aspects of the epigenome, and thus uncover new vulnerabilities that can be leveraged to improve clinical outcomes.

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Acknowledgments: We thank R. Levine, M. Rivera, M. Suva, E. Rheinbay, and B. Knoechel for thoughtful discussions and constructive comments.

10.1126/science.1223730

MATERIALS SCIENCE

Primed to Remember

Dan Hewak and Behrad Gholipour

Human memory can be primed. Expose the brain, even unconsciously, to a word or object, and in subsequent exposures, the memory synapses are faster, and more details are remembered for longer times (1). Why not do the same with the electronic memories in our computers, phones, digital cameras, and game consoles? On page 1566 of this issue, Loke *et al.* (2) show that writing speeds in next-generation electronic memory—ones based on changing a material's phase from a crystal to a glass—have increased substantially through a preswitching incubation process. This priming enabled them to break the 1-ns barrier for electronic switching. On page 1561, Nam *et al.* (3) remarkably provide images and recordings of the electronic changes that occur in memory cells only a few nanometers in size that allow us to watch the switching mechanism. Both of

these studies provide valuable information on the physics of phase-change memory (PCM) and also clues on how this technology could change computer architectures.

The most common nonvolatile memory (one that retains data with the power off) used in laptop computers, smartphones, and video-game consoles is flash memory, which traps a few electrons within an electronic “gate”; the charged state represents a bit of information. However, the size of flash memory cells has been shrunk to near fundamental limits; smaller cells would lose memory information from electrons leaking through the gate into neighboring cells.

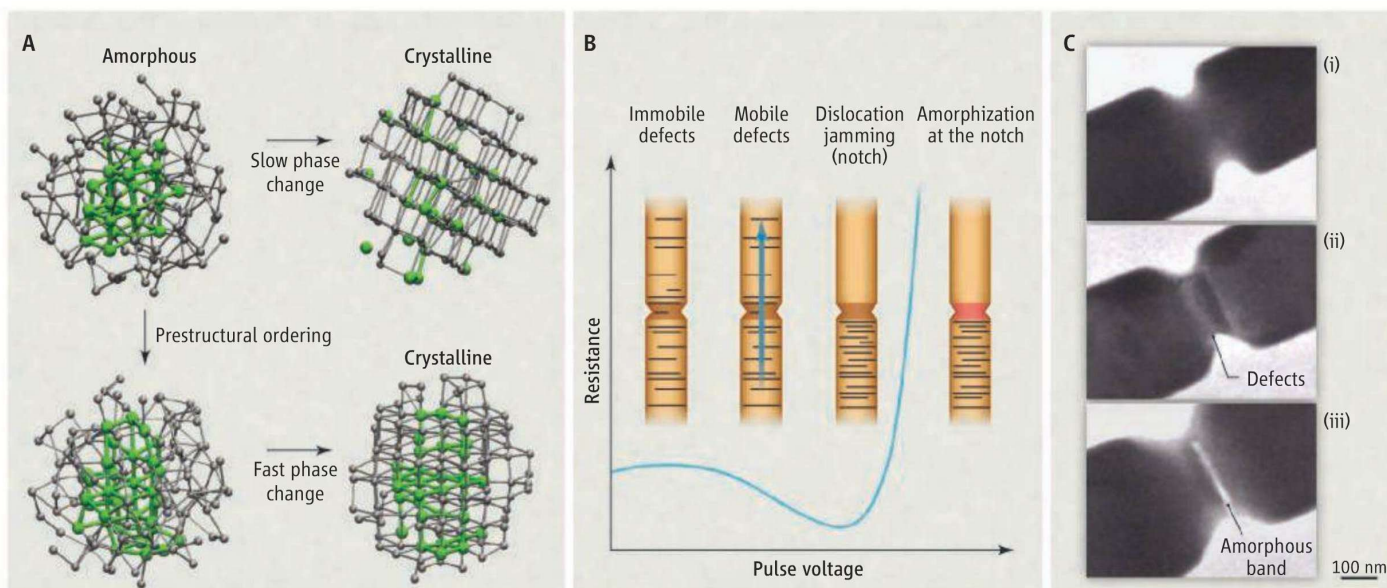
Unlike flash memory, PCM does not depend on trapped electrons, and memory cells can be reduced to much smaller sizes. Phase-change materials for memory applications are typically metallic alloys, commonly based on germanium, antimony, and tellurium ($\text{Ge}_2\text{Sb}_2\text{Te}_5$, or GST). These compounds can easily be switched between the amorphous and crystalline phase, by heating with a laser (as in a DVD or CD disk),

With the potential to mimic the human brain, phase-change memories are operating faster, while imaging provides further insight into the switching mechanism.

or with a small electrical current when the GST is part of an electronic memory chip. Like flash memory, PCM is nonvolatile, and once switched, the phase remains stable until the cell is rewritten. In addition to smaller cell sizes, PCM is inherently faster and can switch repeatedly tens of millions of times, versus only several thousand times for flash memory. Electronic PCM could allow computers to boot instantaneously and greatly enhance the overall performance of computer networks. Many researchers argue that these materials will provide a universal memory technology that could replace both magnetic hard drive and dynamic random-access memory, and even mimic the human brain (4).

Breaking the speed limits of PCM is how Loke *et al.* describe their recent advances. Priming of human memory works best when the two stimuli are in the same modality (e.g., visual priming works best with visual cues). A weak electric current primed the cell, then a short higher-intensity pulse stored the bit of information. This tandem process significantly sped up crystallization;

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Making memories. (A) Model configurations generated by Loke *et al.* of atomic rearrangements during phase transition with or without prestructural orders (priming) that sped up the phase change. (B) This schematic shows dislocation nucleation, transport, and jamming by electrical pulses observed by Nam *et al.*, along with corresponding changes in electrical resistance. Electrical pulses induce heat shocks that nucleate dislocations from atomic vacancies. Larger pulse voltages caused the dislocations to move along the direction of holes. At high dislocation densities, a jamming transition (e.g., in the notch

region or elsewhere for regular wires) occurred as dislocations piled up, and the resistance minimized. Further pulses created an amorphous region and a more insulating phase. (C) A series of snapshots from a bright-field TEM movie by Nam *et al.* in which the as-fabricated nanowire initially shows a uniform dark contrast along the nanowire (i). As electrical pulses were applied, a variation in contrast was seen, with the darker line in the middle of the notch (ii) indicating a region of high strain. This region changed into bright contrast upon amorphization (iii).

switching speeds of 500 ps were reached for the smallest cells. They used computer simulations to help identify a structural origin to this speed increase, which they believe is induced through thermal prestructuring (see the figure, panel A). As measured during this electronic priming, a resistance dip suggests some permanent preswitching structural modification.

The effects of priming the human brain can be imaged by monitoring the brain's frontal region activity using an electroencephalogram (5). In an analogous fashion, Nam *et al.* extend our understanding of the phase-change mechanism by using in situ transmission electron microscopy (TEM) to watch switching directly. By using single-crystal GST nanowires, which provide an open geometry, they viewed the material during the actual switching process (see the figure, panel B). Their direct observation of amorphization in a crystalline phase-change material revealed astonishing insight into the phase-change mechanism. When a voltage was applied across the nanowire, the TEM imaging showed visible contrast changes associated with the now characteristic resistance dip. With a continuously increasing current, defects became mobile and began to propagate along the direction of hole-carrier motion.

At the point of lowest resistance, the movement jammed and a tangled region of highly accumulated dislocations formed, which was followed by switching into an amorphous state. This glassy state appeared as a clear bright line and was confirmed as amorphous by electron diffraction measurements. Nam *et al.* make the analogy of traffic on a highway, in which a simple analytical model predicts a sharp catastrophic jamming transition when the vehicle density exceeds a certain fraction of the maximum packing density (6). In an inspired next step, they created a notch in their nanowire, akin to closing a lane on a busy highway. Defects piled up and an amorphous band appeared at the restriction (see the figure, panel C).

Recently, it has been argued that PCM materials do not change from glass to crystal by melting to a liquid and resolidifying, but rather transform via an all solid-state process. Nam *et al.* may have provided visual evidence of this hypothesis. As Kolobov *et al.* (7) explained, "distortions in the crystalline phase may trigger a collapse of long-range order, generating the amorphous phase without going through the liquid state, upsetting yet another commonly held belief that attributes the change in properties to the loss of long-range order."

Unlike human brains, today's computers deal with processing and memory separately. Data are constantly moved around, resulting in a speed and power "bottleneck." Kuzum *et al.* (8) describe brain-inspired computing and identified phase-change materials as ideal for the implementation of synaptic plasticity. Unlike binary memory applications, they used the continuous transition between resistance levels of phase-change states in an analog manner to emulate biological synapses. Wright *et al.* demonstrated that phase-change materials can both store and process information simultaneously (9) and could be used to make artificial neurons and synapses. Another major hurdle is power consumption; supercomputers consume substantially more energy than the human brain while "thinking" (10). These studies, along with recent new PCM designs by Xiong *et al.* (11), show that there is promise for power reductions through the use of PCM technology.

The studies by Loke *et al.* and Nam *et al.*, along with related work in other labs, should not only pave the way for phase-change memories with ultrafast switching speeds,

low-energy consumption, and reduced memory cell sizes, but also lead to a better understanding of the mechanisms responsible for the phase-change phenomena that could further improve switching speeds. The potential to emulate the human nervous system is gaining increasing attention, as these combined works provide further evidence that phase-change materials could be used to make artificial neurons and synapses.

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10.1126/science.1223365

ECOLOGY

Biotic Multipliers of Climate Change

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A focus on species interactions may improve predictions of the effects of climate change on ecosystems.

Many species face uncertain fates under climate change. Some will persist by shifting their range or adapting to local conditions, whereas others will be lost to extinction. Efforts to lessen the impacts of climate change on biodiversity depend on accurate forecasts. Most studies aiming to identify likely winners and losers consider species one at a time with a "climate envelope" approach that correlates species' occurrences with climatic and environmental variables. Using this method, researchers have predicted that by 2050, 15 to 37% of species will be faced with extinction (1). But which species are most likely to be under

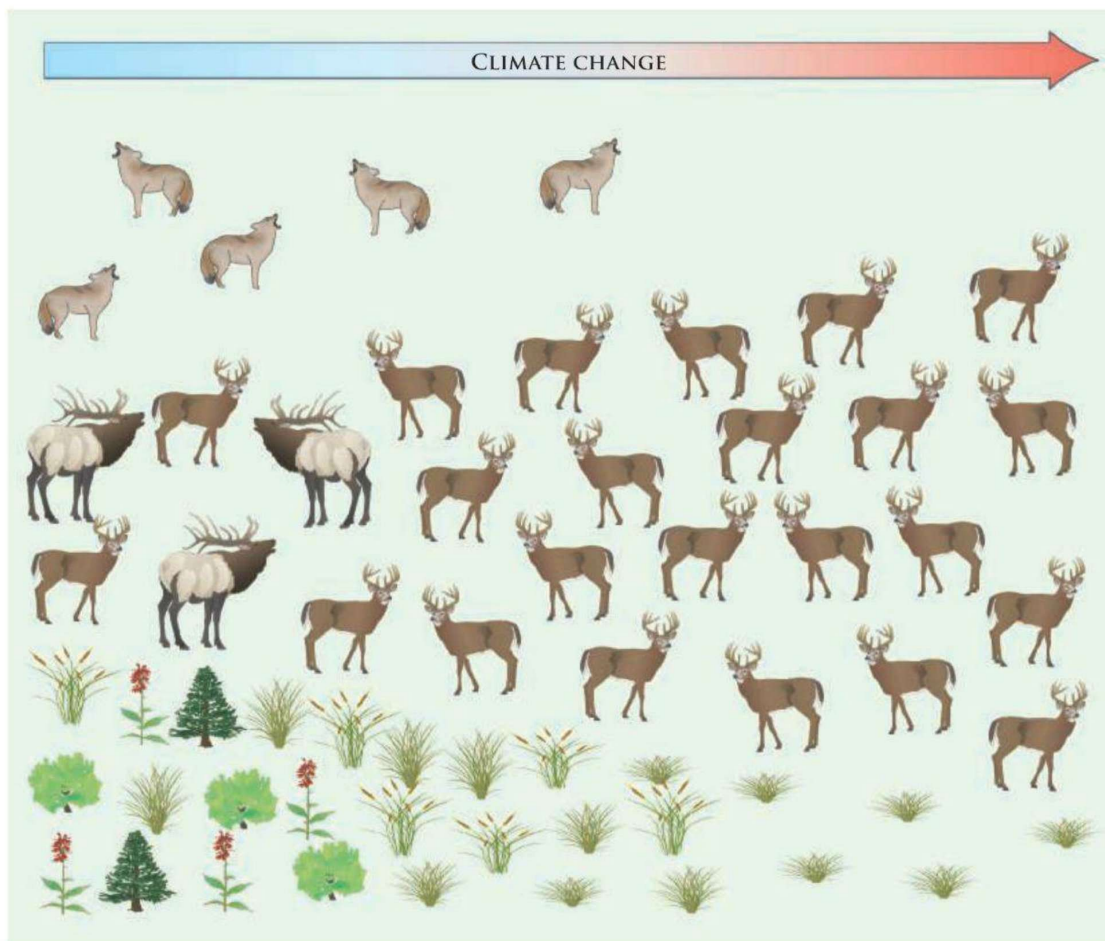
threat? And how will their loss affect the broader ecological community?

The climate envelope approach ignores a core truth of ecology: Species interact with each other in ways that deeply affect their viability. Certain species impart particularly strong effects on others. Consequently, climate change impacts on these species could initiate cascading effects on other species. In effect, these species act as biotic multipliers of climate change. The inherent complexity of species interaction networks has discouraged their consideration in predictions. Emerging research illustrates that trophic interactors are especially strong candidates for biotic multipliers of climatic change. Focusing on these species and their interactions is one path through the complexity.

Recent findings highlight the importance of undisturbed vertical interactions involv-

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Climate change and species interactions. Recent findings suggest that climate change should affect top consumers more strongly, disrupting vertical interactions and thereby affecting many species across trophic levels. In this general example, climate change reduces top predators, leading to an increase in herbivores, and a decrease in plants. As a result, the community experiences an overall decrease in both species diversity and stability.

ing top consumers as climate changes (2). Vertical interactions include those between consumers and their resources (e.g., predator-prey), as opposed to lateral interactions between species in the same trophic level (i.e., interspecific competition).

Why top consumers? Adding or removing top consumers leads to disproportionate changes in community composition across trophic levels (3, 4). Moreover, species in higher trophic positions are more sensitive to changing temperatures (5). Therefore, climate change may have especially strong effects on top-consumer extinctions and range shifts. In turn, these effects can ripple through an entire food web, multiplying extinction risks along the way.

These ideas are supported by insights from recent artificial warming and top herbivore exclusion experiments in Arctic Greenland. The coincident warming of tundra vegetation and removal of caribou and muskoxen herbivores decreased plant species diversity and lowered community stability (2). In contrast,

undisturbed vertical interactions between herbivores and plants promoted community stability by mediating the outcomes of lateral competitive interactions among tundra plants (2). A similar outcome occurred in the paleontological record, where extinctions of large herbivores altered vegetation communities and fire regimes (6).

These destabilizing outcomes are also seen in studies of top predators. On Isle Royale (an island in Lake Superior, USA), rising winter temperatures combined with canine parvovirus produced a trophic cascade: Declining wolf populations caused moose populations to surge and balsam fir to decline (7). In the rocky intertidal of the North American Pacific Coast, higher temperatures led to range contractions in mussel species, exacerbating keystone predation by seastars, which resulted in the decline and local extinction of certain mussel species (8).

These findings contribute to an emerging idea that when climate change disrupts vertical interactions through an increase or

decrease in top consumers, we can expect to see a multiplier effect on many species across trophic levels (see the figure). In the examples cataloged thus far, communities generally become less stable and less diverse. In addition, climate change is expected to compound the risk for top consumers that are already threatened by additional anthropogenic stressors (9).

Although the importance of vertical interactions is well-founded, much remains to be learned. Climate can affect interaction strength and direction in multiple ways (10, 11), including strengthening competitive effects (12) that influence multiple species (13). However, climatic disruption of lateral interactions could affect fewer species than the disruption of vertical interactions because of functional redundancies within trophic levels. Variation in climate might also ameliorate effects by superior competitors and thus promote stability of

the overall community (2).

Though recent models emphasize the need to consider multiple interacting species (14), models should also assess under which conditions vertical versus lateral interactions are important and which local interactions are most likely to scale up to alter regional and global species distribution patterns. Frameworks exist to accommodate different types of species interactions and varying sensitivities to environmental change in a local food web (15). One approach is to vary the relative climatic sensitivity of vertical versus lateral interactions in food web models and examine the consequences for extinctions within local communities. Across broader spatial scales, species interactions can be incorporated into multispecies distribution models via interaction matrices (16), and the biotic multiplier effect can be tracked by following how changes in the abundances of target species (such as top consumers) alter community composition in space and time.

Above all, identifying biotic multipliers will depend on high-resolution biodiversity data to parameterize models and test predictions. With rare exceptions, such community-level data do not currently exist at the temporal and spatial scales necessary to understand climate change impacts. The investments in collecting this needed information would be substantial, but the benefits include forecasting and thus avoiding major losses of species and the services they provide.

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Acknowledgments: This article was inspired by talks by E. Post, M. Araújo, and J. Williams in the symposium, "The future of ecological communities under climate change: no analog?" held at the 2012 AAAS Meeting in Vancouver, BC. The symposium was supported by the Yale Institute for Biospheric Studies. We thank the speakers and O. Schmitz for helpful comments. Support came from the Yale Climate and Energy Institute (P.L.Z.), NSF award DEB-1011335 (D.K.S.), and NSF award DEB-1119877 (M.C.U.)

10.1126/science.1222732

ATMOSPHERIC SCIENCE

Carbon from Tropical Deforestation

Daniel J. Zarin

How much carbon is emitted from tropical deforestation? Attempts to answer this question have generally relied on data from national inventories. More recently, sufficient satellite data have become available to provide independent estimates. On page 1573 of this issue, Harris *et al.* (1) report a global estimate of tropical deforestation emissions derived entirely from satellite data. For the period from 2000 to 2005, those emissions are much lower than previously reported.

In 2007, the Intergovernmental Panel on Climate Change (IPCC) concluded that the "best estimate" of net carbon emissions from tropical land use change in the 1990s was 1.6 ± 0.6 petagrams of carbon per year (Pg C year^{-1}), equivalent to $\sim 20\%$ of greenhouse gas emissions from human activities during that decade (2). That and most other estimates have relied to varying degrees on national self-reporting to the Global Forest Resources Assessment of the United Nations Food and Agricultural Organization (FAO) (3). However, the quality of those data are uneven (4), the reported extent of forest cover and deforestation differs from that found in satellite surveys (5), and the forest carbon estimates are based on a broad set of assumptions (3). Satellite-based analyses of forest cover have since improved estimates of the extent of deforestation across the tropics (6, 7), but satellite data that are sufficient for the task of estimating forest biomass, and hence carbon stock ($\sim 50\%$ of biomass), have only recently become available.



Gross deforestation emissions. Harris *et al.* provide an independent benchmark for national gross deforestation emissions for 2000 to 2005 [see table S2 in (1)], shown here for the top five emitting countries (peat emissions not included). The 90% prediction interval around those emission estimates is substantial. To reduce that uncertainty, the authors suggest using higher-resolution satellite data to estimate forest cover loss at the country level. DRC, Democratic Republic of Congo.

In the past year, two groups have independently published maps of tropical forest carbon stocks based on multisensor satellite data calibrated with ground measurements. Saatchi *et al.* (8) estimated those stocks at 247 Pg C . Using a different methodology, Baccini *et al.* reported a remarkably similar estimate of 228.7 Pg C (9). They also used their map, spatial information on the location of deforestation (6), and the FAO data (2) to generate an estimate of net carbon emissions from tropical deforestation of 1.0 Pg C year^{-1} for the period from 2000 to 2010.

Harris *et al.* now assess gross carbon

emissions from tropical deforestation—as opposed to net emissions, which include forest regrowth—without resorting to the FAO data. (Gross carbon emissions from deforestation are defined as the area of gross forest loss multiplied by the carbon stock of the forest before clearing.) The authors use satellite-based analyses of the geographic distribution of tropical forest carbon stocks (8) and of both the location and quantity of tropical deforestation (6, 7). The study overcomes a mismatch in the spatial scales of the carbon stock and deforestation maps through a repeated, randomized statistical sampling

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methodology that provides a robust estimate and an associated range of uncertainty.

Harris *et al.* obtain a comparatively low gross emission estimate of 0.81 Pg C year⁻¹ for the period between 2000 and 2005; the equivalent value from Baccini *et al.*'s analysis is 2.22 Pg C year⁻¹ (9). Neither estimate includes globally significant emissions associated with the loss of carbon-dense tropical peatlands, mostly from Indonesia, where such losses are responsible for roughly half of all greenhouse gas emissions (10). The large difference between the two estimates (1, 9) may result from definitional and methodological issues; the explanation is not immediately apparent, and the magnitude of the difference remains surprising, particularly given the similarity between the underlying forest carbon stock data.

A difference of this magnitude in tropical deforestation emissions estimates between two state-of-the-art analyses is also cause for concern in climate policy circles. Avoiding a >2°C increase in global average temperature becomes more difficult with each passing year, but it is still achievable if greenhouse gas emissions from all sources peak in 2020 at a rate equivalent to ~3.3 Pg C year⁻¹ below "business-as-usual" projections (11). In that context, it would benefit both the science and policy communities if the two research groups (1, 9) could determine the reasons for the 1.41 Pg C year⁻¹ difference in their results soon, and do so with sufficient transparency for others to evaluate.

Within the United Nations Framework Convention on Climate Change (UNFCCC), negotiators have agreed to the voluntary establishment of benchmarks for assessing developing country performance in reducing emissions from deforestation and forest degradation (REDD+) (12). Developing countries have been invited to submit REDD+ reference emission levels, taking into account historical data and IPCC guidance and guidelines (12). Interested countries may wish to consult Harris *et al.*'s independent benchmark for national gross deforestation emissions for the period from 2000 to 2005 [see the figure; for full data, see table S2 in (1)] as input to their UNFCCC reference emission level submissions (11).

Parallel to the UNFCCC negotiations, the government of Norway has been paying out on a US\$1 billion commitment to Brazil, at a rate of US\$5 per ton of CO₂, for deforestation emission reductions on the basis of the Brazilian Space Agency's annual reporting on Amazon deforestation, which has been falling since 2005 (13). The Norwegian Government has committed another US\$1 billion to

do likewise in Indonesia (14), if a legitimate monitoring system is developed there (which seems plausible) and if Indonesia's emissions are reduced (which remains uncertain).

Independent monitoring against reliable deforestation emission benchmarks, like those reported by Harris *et al.*, would help to underpin the integrity of these and other "payment for performance" REDD+ arrangements and provide an unbiased assessment of tropical deforestation emission reductions. The science is getting close to achieving that. Comparisons between different approaches (1, 9) and different scales of analysis (15) could get us even closer and should not be delayed. Estimates of more recent emissions from tropical deforestation that are methodologically consistent with the 2000 to 2005 benchmarks would address a critical need for objective quantification of deforestation emission changes since 2005.

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10.1126/science.1223251

MOLECULAR BIOLOGY

Wnt Regulates TERT—Putting the Horse Before the Cart

Carol W. Greider

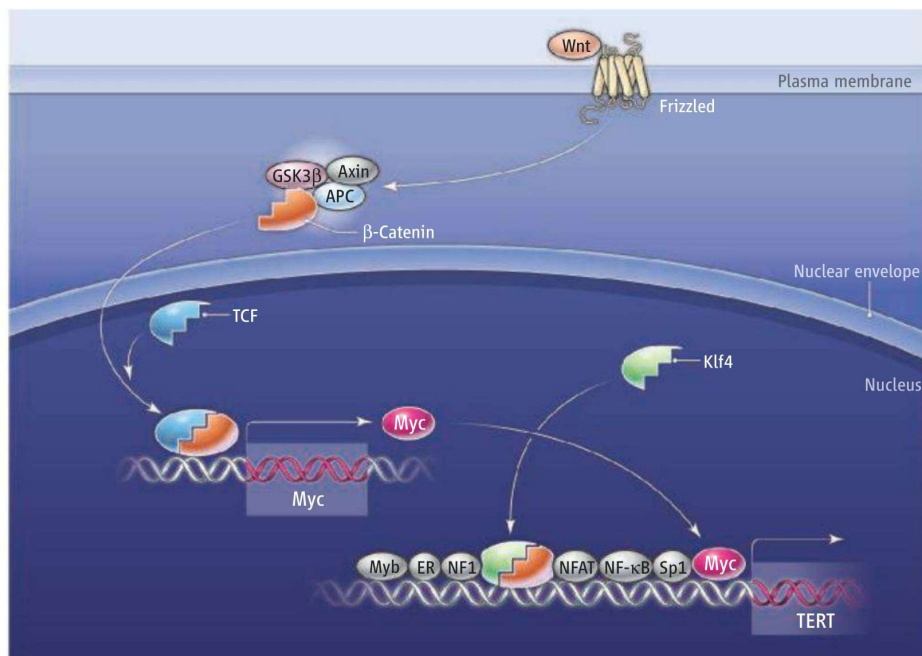
A cell signaling pathway that controls self-renewal also regulates telomerase activity.

Maintaining the length of telomeres—the ends of chromosomes—is essential for all cells that divide many times. The enzyme telomerase lengthens these ends, counterbalancing their shortening that occurs each time chromosomes are copied. Telomerase is essential for cell viability, and loss of its function from the loss of only one of two copies of the encoding gene can lead to the failure of stem cell renewal that is seen in premature aging conditions such as dyskeratosis congenita, aplastic anemia, and pulmonary fibrosis (1). Conversely, telomerase

activity is increased in many cancers and may be required for cancer cells to maintain their telomere length (2). On page 1549 in this issue, Hoffmeyer *et al.* (3) show that a cell signaling pathway known to play a prominent role in development, stem cell renewal, and cancer (4)—the canonical Wnt pathway—also regulates the expression of telomerase.

Activation of the canonical Wnt signaling pathway in animal cells occurs upon binding of the secreted protein Wnt to a frizzled receptor at the plasma membrane (see the figure). Frizzled signals through the glycogen synthase kinase 3β (GSK3β) kinase, which inactivates a destruction complex containing the proteins adenomatous polyposis coli (APC) and Axin. This stabilizes the protein β-catenin, enabling it to translocate to the

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A place at the promotor. Binding of Wnt ligand to a frizzled receptor at the cell surface activates the GSK3 β kinase, which blocks destruction of β -catenin by the complex containing Axin and APC. Stable β -catenin can then enter the nucleus and, together with coactivators, activates a set of genes including *c-Myc* (with coactivator TCF4). The *c-Myc* protein can activate the *TERT* promoter itself, which may contribute to TERT activation by β -catenin. The *TERT* promoter is a very crowded site with many different transcription factors that bind to it. Klf4/ β -catenin coactivator complex now must squeeze in to the crowded promotor.

nucleus, associate with specific DNA binding proteins, and activate the transcription of a defined set of genes (4). The Wnt pathway has previously been shown to activate telomerase (5), and mutations in APC (6) and GSK3 (7), important regulators of the Wnt pathway, also increase telomerase activity. Hoffmeyer *et al.* further analyzed the pathway through which Wnt signaling activates the enzyme. Telomerase is a specialized reverse transcriptase; the enzymatic subunit (TERT) uses the RNA component (TR) as a template to synthesize telomere repeats onto chromosome ends (8). The authors found that *TERT* transcription is substantially reduced in β -catenin-deficient mouse embryonic stem cells and in adult stem cells. By expressing a constitutively stable form of β -catenin, Hoffmeyer *et al.* show that in vivo, β -catenin expression increases *TERT* transcription. The effect is direct because β -catenin binds to the *TERT* promoter, as shown by reporter assays and chromatin immunoprecipitation. This is in contrast to indirect activation of *TERT* transcription by β -catenin, in which the transcription factor *c-Myc* directly controls *TERT* transcription (9) though *c-Myc*, which itself is transcriptionally regulated by β -catenin. Furthermore, the authors show that the protein Klf4—rather than the usual suspect for a β -catenin DNA binding partner, T cell factor 1–4 (TCF1–4)—cooperates with

β -catenin to induce *TERT* expression. This puts a new twist on regulation by the Wnt signaling pathway. Klf4, like β -catenin, is a master regulator of stem cell fate; it is one of the four factors that induce the pluripotent state in induced pluripotent stem cells (10).

Because of the importance of telomerase expression, the signaling pathways that control *TERT* transcription have been extensively studied. Remarkably, many different transcription factors, including *c-Myc*, Sp1, nuclear factor of activated T cells (NFAT), activating protein 2B, nuclear factor κ B (NF- κ B), Myb, activating transcription factor, nuclear factor 1 (NF1), and the estrogen receptor (ER), bind to the 330–base pair minimal *TERT* promoter and regulate transcription. In addition, a number of negative regulators bind the *TERT* promoter, including CTCF, elongation factor 2, p53, Ets, Mad1, Men1, and Wt1 (11). Adding β -catenin and Klf4 to the many regulators that bind the *TERT* promoter is like adding one more guest to a crowded table at a dinner party.

Why are so many regulators needed for a gene thought to not be expressed in many human cells? The amount of telomerase expressed must be very important to those cells that need it, because genetics shows that having half the amount of either the telomerase RNA component or TERT leads to devastating degenerative disease in humans (1).

Perhaps the dinner table is not as crowded as it might seem if different transcriptional regulators are used by different cell types to respond to specific growth signals and finely tune the expression of telomerase to establish the telomere-length equilibrium.

Recent studies have proposed that the Wnt pathway is linked to TERT in a quite different way. Constitutive overexpression of TERT in mice activates the Wnt pathway, suggesting that TERT may also function as a transcription factor (12). Although one study did not observe Wnt pathway activation in response to TERT overexpression (13), other studies have raised questions about the physiological relevance of the constitutive overexpression of TERT. Deletion of TERT in mice does not affect expression of target genes in the Wnt pathway (14), nor give rise to the cellular phenotypes that loss of Wnt signaling induces (15), indicating that TERT regulation of Wnt signaling may be limited to situations where TERT is overexpressed.

It is reasonable to propose that Wnt regulates *TERT* given that Wnt signaling plays an essential role in stem cell self-renewal and that TERT is needed for the long-term growth of stem cells. *TERT* regulation seems to require not one, but two master transcriptional regulators to assure that there is neither too much, which may allow the growth of cancer cells, nor too little, which might lead to stem cell failure. The finding by Hoffmeyer *et al.* that both β -catenin and Klf4 are required to activate *TERT* expression puts the horse (Wnt) before the cart (TERT) and provides a foundation for linking telomerase levels and self-renewal.

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INTRODUCTION

H5N1

THE PUBLICATION IN THIS ISSUE OF THE RESEARCH PAPER *AIRBORNE TRANSMISSION of Influenza A/H5N1 Virus Between Ferrets*, plus its newer companion *The Potential for Respiratory Droplet-Transmissible A/H5N1 Influenza Virus to Evolve in a Mammalian Host*, marks the end of more than 8 months of widely reported controversy over whether some of the data now freely accessible should be withheld in the public interest (see http://scim.ag/H5N1_Flu for a compilation of News and Commentary recently published in *Science*). As a result, people worldwide are now much more aware of the potential threat that this virus, commonly known as “bird flu,” poses to humanity. And the open publication of new data concerning the potential of H5N1 to convert directly to a form that can be transferred through the air between ferrets will motivate many more policy-makers and scientists to work to reduce the likelihood that this virus will evolve to cause a pandemic. Breakthroughs in science often occur when a scientist with a unique perspective combines prior knowledge in novel ways to create new knowledge, and the publication of the two research Reports in this issue will hopefully help to stimulate the innovation needed, perhaps from unsuspected sources, to make the world safer.

As described in News and Commentary pieces in this special section, the prolonged controversy has also provided a “stress test” of the systems that had been established to enable the biological sciences to deal with “dual-use research of concern” (DURC): biological research with legitimate scientific purposes that may be misused to pose a biologic threat to public health and/or national security. One centerpiece of this system is the U.S. National Science Advisory Board for Biosecurity (NSABB). *Science* strongly supports the NSABB mechanism, which clearly needs to be supplemented and further strengthened to deal with the inevitable future cases of publication of dual-use research, both before and after their submission to journals. Still missing is a comprehensive international system for assessing and handling DURC—one that provides access, for those with a need to know, to any information deemed not to be freely publishable.

If fields subject to DURC are to attract the outstanding young scientists required to address problems such as those posed by H5N1, the appropriate experts may need to define in advance the most promising research strategies and, acting in concert with security experts, agree on responsible ways to address them. It is our hope that the thoughtful Commentaries, News, and research Reports in this special issue will help to jump-start intensive efforts along these lines.

— BRUCE ALBERTS
Editor-in-Chief of *Science*

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Science

Benefits and Risks of Influenza Research: Lessons Learned

Anthony S. Fauci* and Francis S. Collins

Given the yearly challenge of seasonal influenza and the potential catastrophic consequences of future pandemics, the need for intensive basic and clinical influenza research is unquestionable. Although the fruits of decades of research have enabled dramatic improvements in our ability to prevent and treat influenza, many fundamental questions remain, including those related to the complex factors associated with host switching and transmission of influenza viruses. Recent public concern over two H5N1 influenza manuscripts that studied the transmissibility of influenza viruses has triggered intense discussion on dual-use research and the way forward.

Influenza A virus is an ancient and persistent threat to individual and global health (Fig. 1). Seasonal influenza (which occurs annually, usually in winter) kills ~500,000 people globally and up to 50,000 people in the United States each year. Influenza viruses have animal reservoirs, especially in birds and pigs. They can undergo extensive genetic changes and even jump species, sometimes resulting in a virus to which humans may be highly vulnerable. Such an event can lead to a global health disaster; global influenza pandemics have occurred only three times in this past century. A prime example is the 1918 influenza pandemic, which killed between 50 and 100 million people worldwide and caused enormous social and economic disruption. Influenza A viruses circulate widely and are constantly evolving toward pandemic capability, as seen again in 1957, 1968, and 2009 (*1*). There is thus a clear danger of future pandemics.

Over the last decade, a highly pathogenic avian influenza virus (genus A; subtype H5N1) has emerged among chickens (*2*). Rarely, the virus has spread to humans, usually individuals with heavy exposure to infected birds. Since 2003, ~600 confirmed cases have occurred in humans in more than a dozen countries. Nearly 60% of these reported cases have resulted in death (*3*). Because it has been impossible thus far to completely eliminate the virus from chicken flocks or from wild birds or to prevent transmission to mammals, there is a persistent danger that H5N1 viruses [which have continually mutated and evolved (*2*)] will eventually become more easily transmissible to and among humans. Because humans are not specifically immune to H5N1 influenza, this scenario would have the makings of a potentially devastating pandemic.

One of the goals of pandemic influenza research is to recognize and anticipate how viruses are evolving in the wild toward a phenotype that

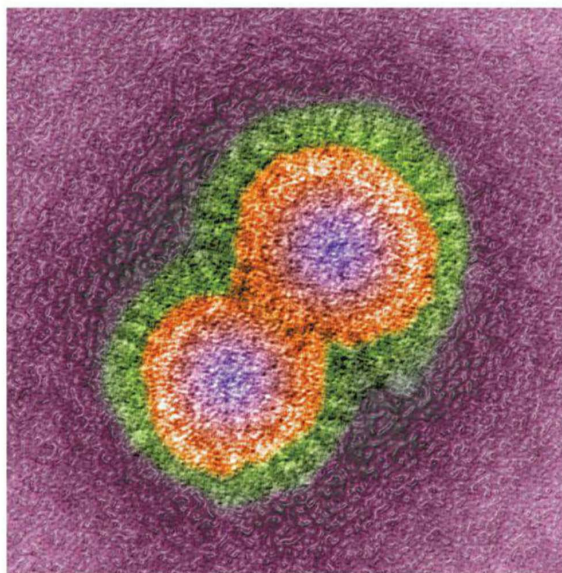


Fig. 1. H5N1 avian influenza virus particles, colored transmission electron micrograph. Magnification: $\times 670,000$ when printed 10 cm wide.

is dangerous to humans, thereby staying one step ahead of potential pandemics. In this regard, compelling research questions relevant to global health and pandemic preparedness include determining whether highly pathogenic viruses, such as H5N1, have the ability to mutate and/or reassort with another influenza virus to become readily transmissible by the airborne route among humans. If so, (i) what is the likelihood that such mutations or reassortments will happen in nature? (ii) Is there a genetic signature of such a virus that might be helpful in surveillance? (iii) Would such a virus be highly pathogenic for humans? And (iv), would such a virus be sensitive to currently available antiviral drugs and vaccines, or would new ones be necessary? In response to these and related questions, the National Institutes of Health (NIH) has intensified the research we conduct and support on pandemic influenza. Much of this research is specifically focused on developing

improved countermeasures, including a “universal” influenza vaccine that would protect people from multiple influenza subtypes. In complementary research, NIH-supported scientists study the factors involved in the pathogenesis and transmissibility of H5N1 and other influenza viruses to nonhuman mammals (mice, guinea pigs, ferrets, and nonhuman primates) to identify potential clues to the determinants of the same properties in humans.

Within this context, global attention has been paid recently to two NIH-funded studies of H5N1 transmissibility and pathogenesis in ferrets. In those studies, H5N1 viruses were made transmissible via respiratory droplets among ferrets by engineering the virus; well-described and published protocols including reverse genetics, reassortment, and passaging of viruses in mammals were used. Manuscripts describing the studies (*4, 5*) have generated an unprecedented degree of discussion, concern, and disagreement among scientists, as well as the public, regarding whether the experiments should have been performed in the first place and whether they should be published in their entirety. Major sources of concern have been that the results might be used by bioterrorists to harm the public or that the virus might accidentally escape and cause a pandemic.

Research on H5N1 viruses, including the experiments reported in these two papers (*4, 5*), is comparable to that which has been conducted over decades with other seasonal and pandemic influenza viruses in ferrets and other animal models. The use of the ferret as an animal model for influenza transmissibility dates back to the 1930s,

as ferrets are easily infected with influenza and sneeze when infected, which is useful for studying the airborne route needed for sustained human-to-human transmission. Understanding the virus characteristics associated with enhanced transmissibility—even in an imperfect animal model, such as the ferret—can benefit surveillance for naturally evolving wild viruses if they continually mutate toward a “genomic signature” that could be recognized as potentially predictive of a certain phenotype. Given the complexities of viral transmission, a virus’s ability to adapt to a host species, pathogenesis (a virus’s ability to cause disease), and the interrelation among these factors, which are likely to be unique to each influenza virus, any particular genomic signature will not necessarily predict how a given virus will act. Nonetheless, studies such as these provide incremental knowledge that the scientific community can build upon. A more in-depth

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understanding of the genetic evolution of influenza viruses should positively affect our ability to recognize and respond to influenza outbreaks.

However, whenever one deliberately manipulates a virus or a microbe, it is always possible, at least theoretically, that the research results could be used by bioterrorists to intentionally cause harm, or that an accidental release of a pathogen from a laboratory could inadvertently cause harm. Such research is referred to as “dual-use research,” as the research potentially has both positive and negative applications. A particular subset of dual-use research is referred to as “dual-use research of concern” or DURC. DURC is defined as life sciences research that, on the basis of current understanding, can be reasonably anticipated to provide knowledge, information, products, or technologies that can be directly misapplied to pose a significant threat with broad potential consequences to public health and safety, agricultural crops and other plants, animals, the environment, materiel, or national security (6). If a particular experiment is identified as DURC, that designation does not inherently mean that such research should be prohibited or not widely published. However, it does call for us to balance carefully the benefit of the research to public health, the biosafety and biosecurity conditions under which the research is conducted, and the potential risk that the knowledge gained from such research may fall into the hands of individuals with ill intent. Research that could enhance the transmissibility of H5N1 viruses clearly is DURC.

In this regard, the question of whether to publish the two H5N1 studies in ferrets has been intensively discussed by an independent federal advisory committee known as the National Science Advisory Board for Biosecurity (NSABB) (7, 8). On the basis of their recommendations and other evaluations, the U.S. government agreed that the research is important for the public health and should be published. However, important lessons were learned along the way and, appropriately, triggered an examination of our approach concerning the conduct, oversight, and communication of DURC. In this regard, the U.S. government announced on 29 March 2012 the U.S. Government Policy for Oversight of Life Sciences Dual Use Research of Concern (6). This policy document outlines, for federal departments and agencies that conduct or fund life sciences research, steps to determine whether projects fall under the definition of DURC, to assess the risks and benefits of these projects, to review them regularly, and to develop risk mitigation plans. In the process of weighing the potential risks and benefits of publishing these two manuscripts (4, 5), it also became clear that, when possible, it is critical to identify research with DURC potential before the initiation of the project and, certainly, before the results are submitted for publication. Such monitoring in the case of NIH-

funded research requires the concerted effort of all involved, including scientists applying for or in receipt of NIH funding and NIH program officials. Additional guidelines will be needed as well to assist biosafety committees in evaluating DURC at the institutions where the research is conducted.

Furthermore, as a result of the public discussion of these two manuscripts, major gaps in our knowledge of influenza became painfully obvious. For example, there was considerable scientific debate about how well data from the ferret model can be extrapolated to understand influenza virus transmission and pathogenesis in humans. An H5N1 virus strictly adapted for ferret transmissibility may not be entirely relevant to humans. Moreover, although it is likely that the officially reported 60% case-fatality rate for human H5N1 influenza is artificially high (because nonfatal cases are less likely to be reported), there are limited surveillance data on which to base a more accurate estimate. NIH has begun a dialogue with the influenza research community about addressing these and other questions and will initiate a more strategic approach to defining the research gaps that must be addressed in order to responsibly move the field forward. In addition to identifying research gaps, the discussion of these manuscripts underscores the important practical issues of implementing rapid turnaround time between virus isolation and sequencing to provide real-time surveillance.

Finally, despite the importance of performing influenza research that may have DURC potential, this recent experience has underscored the fact that civil society needs to be involved in the dialogue early on. Clearly, research should be conducted and published only if the potential benefits to society outweigh the risks to national

security and the potential harm to society. The risk/benefit calculation for certain experiments and their communication is not always obvious, and the current experience reflected considerable disagreement even in the scientific community. The ultimate goal of the new U.S. government-wide DURC policy is to ensure that the conduct and communication of research in this area remain transparent and open and that the risk/benefit balance of such research clearly tips toward benefitting society. The public, which has a stake in the risks and the benefits of such research, deserves a rational and transparent explanation of how decisions are made. It is hoped that the upcoming dialogue related to the new DURC policy will be productive. A social contract among the scientific community, policy-makers, and the general public that builds trust is essential for success of this process.

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10.1126/science.1224305

PERSPECTIVE

Regulating the Boundaries of Dual-Use Research

Mark S. Frankel

A new U.S. policy for dual-use life science research defines what is permissible by scientists and the government. However, further negotiations will be needed as governments realize the consequences of such boundaries for research and society.

The recent furor surrounding H5N1 influenza research and the intervention of national and international governmental bodies into the publication process (1) have

brought to the fore a long-standing question regarding the relationship between science and society. That is, what is the proper role of government in regulating science? The science-society relationship has evolved from a time when science was seen as best left to the scientists to the current environment in which scientists are subject to competing claims from an expanding number of stakeholders who see the relevance

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of science to their core concerns—businesses seeking a profit, universities seeking federal and state funding, patients seeking cures, and politicians seeking votes, among others. As such, there has been an increasing demand for accountability on the part of the scientific community. Toward this end, what should governments do?

The responsibilities associated with being a scientist can be viewed as two basic types. One is internal to science, which addresses the responsibility to adhere to accepted standards of scientific practice when conducting and reporting research. The other goes beyond the responsibilities related to doing science, focusing more on the social consequences of applying research findings. The H5N1 case, in which two research papers (2–4) show that the virus can mutate into a form that might spread rapidly among humans, presents a very compelling challenge for scientists. The controversy over whether to publish the papers intersects with both types of responsibilities. For the first type, it raises questions about how much of a researcher's methods should be readily accessible for others to assess the integrity of the research and its relationship to the published findings, which go to the core of what the scientific community considers to be acceptable research practices. It also raises questions about the scope of scientists' social responsibilities when the research has dual-use implications—offering knowledge that could contribute to greater resilience in the face of a potential pandemic, as well as be intentionally misused or accidentally released with potentially catastrophic effects. The H5N1 case also illuminates scientists' responsibility to follow appropriate biosafety practices to safeguard fellow researchers, as well as the public. World-leading H5N1 researchers agreed to a voluntary, temporary moratorium on research to allow for international discussion (5).

For governments, the challenges of responding to the H5N1 controversy are also compelling. They must deal with increasing public calls for greater government oversight of research, while at the same time relying on scientists to help them prepare for a pandemic disaster. They must also avoid overregulation out of concern that their actions will impede discoveries and innovation. Determining the level of regulation that is “just right” is a balancing act among many competing interests. What the history of the science-society relationship reveals is that the result is an outcome of ongoing negotiations by a range of stakeholders to determine the authority, jurisdiction, and responsibilities—setting the boundaries—of what is or is not permissible action by scientists and the government (6).

The debate over the two research papers led to a resolution regarding their publication (7) in which one paper was published in May by *Nature* (3) while the other is published in this issue of *Science* (4), both in updated, uncensored

forms. However, that does not end the negotiations. At a recent hearing before the United States Senate, called primarily in response to the H5N1 research, Senator Joseph Lieberman (I-CT) remarked that “Although this particular issue appears to have been resolved, it's going to recur and we can't just ‘kick this can down the road’ and deal with it on an ad hoc basis when it happens again” (8). This is a reminder that while the focus of attention has been on the two papers, there is a larger landscape for which boundaries have yet to be negotiated.

One attempt to draw those boundaries in the United States is the new U.S. Policy for Oversight of Life Sciences Dual Use Research of Concern, designed “to establish regular review of United States Government funded or conducted research with certain high-consequence pathogens and toxins for its potential to be dual use research of concern (DURC)” (9). The Policy lists as a principle that the “Government will facilitate the sharing of the results and products of life

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sciences research conducted or funded by United States Government agencies,... In executing this Policy, the United States Government will abide by and enforce all relevant Presidential Directives and Executive Orders,... (9). On the surface, that's good news for science, since current U.S. policy for “sharing of the results and products” of research relative to national security is governed by a Presidential National Security Decision Directive (NSDD-189) issued in 1985 that fundamental (i.e., “basic”) research will remain “unrestricted,” or if national security concerns require control, the mechanism for doing so will be to classify the research (10). However, if one reads further in the new U.S. Policy, the boundaries are less clear. The Policy gives the federal government the responsibility

to consider “risk mitigation measures,” including the following: “Determining the venue and mode of communication (addressing content, timing, and possibly the extent of distribution of the information) to communicate the research responsibly.” If such measures are not adequate, the government will determine whether to “classify the research” or “Not provide or terminate research funding.” The possibility of having one's research classified or funding terminated after the research is underway is not conducive to good science and may well discourage some researchers from engaging in such work.

The U.S. government's policy is a boundary-changing document in its relationship with U.S.-based researchers engaged in dual-use life sciences research, setting aside a previous approach that had essentially deferred to the research community (i.e., scientists, their institutions, and journal editors) to decide what, how, and when research is published. But what does this mean for international research?

The Policy does apply to U.S. scientists in research partnerships with non-U.S. scientists, which could put those collaborations under a cloud of considerable uncertainty, if not in jeopardy. It could also put U.S. science at a disadvantage in competing for international prominence. The Policy does acknowledge the need to pursue “engagement with our international partners” but offers no details. There will be much boundary negotiation, as governments jockey over how policy differences, where policies exist at all, will be reconciled. One troubling example from the H5N1 incident is that the Netherlands required the authors of one of the disputed papers (4) to apply for government permission to submit their paper for publication, claiming that the study falls “under regulations that control the export of weapons technology” (11). That view of the research would appear to be in conflict with the U.S. NSDD-189, as well as U.S. export control regulations, which exempt basic research. But, then, boundaries are often fuzzy and subject to interpretation, and which ones will prevail is often unpredictable. In this case, the Netherlands government viewed the H5N1 research as applied, not basic, and although the authors contested this view, they applied (and received) an export license (12).

What dual-use life sciences research will look like in the U.S. and whether, how, and when it might be disseminated are still open to boundary

definition. Toward that end, governments should agree on a core set of principles that reflect a global consensus on fostering international cooperation, followed by efforts to harmonize policies and practices, including those pertaining to the dissemination of dual-use research, to mitigate the potential for malevolent uses of dual-use research. The scientific community cannot afford to be bystanders in these efforts. This is not merely a matter of self-interest. Scientists have a social responsibility to inform the scientific community, the public, and policy-makers of the potential dangers of their work, as well as of the risks and lost opportunities associated with restricting the flow of scientific informa-

tion. There may be good reasons for governments to control dissemination, but they should understand what the consequences may be for science and policy.

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10.1126/science.1221285

POLICY FORUM

Implementing the New U.S. Dual-Use Policy

Carrie D. Wolinetz

After a decade of intensive policy discussions on the topic of dual-use research of concern (DURC) in the life sciences, there has been a lack of consensus on how to practically define DURC; whether it is feasible to identify and regulate DURC experiments; how to address the risks associated with DURC; and how to balance this risk with the necessity of fostering life sciences research for public health and biodefense. The publication of two avian influenza studies has brought the DURC issue back into sharp focus and has resulted in a new set of federal guidelines. However, the new DURC policy raises questions regarding whether this is the best policy solution to a complicated biosecurity concern.

Since the publication of a 2001 experiment synthesizing polio virus de novo received national attention, the research community has been engaged in a philosophical and policy debate over how to deal with the challenge of dual-use life science research. Dual-use research of concern (DURC) is roughly defined as research that is intended for legitimate, beneficial purposes but also carries a risk of being misused for malicious purposes. The ability to define DURC in a way that facilitates its identification and regulation has been an issue that the biosecurity community has struggled with for nearly a decade (1). Mitigating the risks associated with biological DURC has been the subject of two National Academies reports and major international fora, including meetings hosted by the InterAcademy Panel and associated with the Biological Weapons and Toxins Convention (BWTC). These led to the formation of the National Science Advisory Board for Biosecurity (NSABB), a U.S. federal government advisory committee that has produced multiple reports and workshops in the 8 years since its inception (1). However,

there is still no consensus on how to practically define DURC; whether it is feasible to identify and regulate DURC experiments; how to address risks associated with DURC; and how to balance this risk with the necessity of fostering life sciences research for public health and biodefense.

Recent public attention on the publication of two avian (H5N1) influenza studies has brought the dual-use issue into sharp focus and has resulted in a swift response from the U.S. government in the form of the “United States Government Policy for Oversight of Life Sciences Dual Use Research of Concern” (2). The policy calls on federal agencies to review research involving 15 agents from the select agent list, determine whether they meet the definition of DURC, conduct a risk assessment, and then mitigate risks in collaboration with the institution and scientist conducting the research. It was issued in unusual fashion by posting on the National Institutes of Health (NIH) Office of Biotechnology Activities’ Web site on 29 March 2012 and has raised questions in the research community about whether it is the best policy solution to a complicated biosecurity concern.

Does the Policy Fully Address Dual Use?

The DURC policy is limited to experiments involving 15 agents that are already on the select

agent list (which includes roughly 80 agents). This list was initially generated as part of the federal Select Agent Program established under the Public Health Security and Bioterrorism Preparedness and Response Act of 2002.

This raises a number of questions about the redundancy of the policy. Experiments involving select agents are already stringently regulated by a system that includes background checks on all personnel involved and licensing of the facilities (3). In addition, the Select Agent Rule is currently under review (4), and the proposed changes to the regulations add even more controls to the agents addressed by the DURC policy. Many institutions that conduct a substantial amount of research involving select agents have incorporated this research into biosafety review systems that take place at the local level through Institutional Biosafety Committees (IBCs).

Moreover, many well-documented case studies of DURC that have been cited by the NSABB and many other organizations as evidence for the need for additional DURC guidance or regulation would not be covered by this policy. The de novo synthesis of polio virus, the Australian mousepox experiment, and the Penn State aerosolization study (5)—none of these notorious DURC cases would have been regulated under the new policy.

To the U.S. government’s credit, this policy clearly tries to limit the scope to prevent the over-identification of legitimate research that does not pose much of a risk and to limit the associated burden on research institutions. However, the redundancy with the Select Agent Program and its inherent failure to capture experiments that are commonly agreed to be DURC raises the question of whether it addresses the DURC issue at all. This is the very quandary identified in a 2007 Congressional Research Service report that explored the challenges of defining DURC for the purposes of oversight (6).

Is the DURC Policy Feasible?

The recent review and re-review of the H5N1 avian influenza publications by the NSABB illustrates the difficulty in making decisions about

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how to mitigate the risk of DURC, even as it underscores the need for a workable system to help address the real risks associated with some biological research. The decision-making process ultimately took nearly 5 months and an international summit of experts (7), and the group failed to reach consensus in its final decision to recommend publication (8). Given that this review process dealt with only two publications documenting similar experiments, it is difficult to imagine an analogous review and risk mitigation system applied to a larger body of research not causing delays and disagreements or resulting in an onerous burden for both the agency and the institution.

There are a number of questions raised by both this example and the ambiguities in the policy itself. Who at the federal departments and agencies conducts “a review to identify all current or proposed” research for DURC potential (2)? What expertise must they have? While a timeline for reporting these findings to the U.S. government has clearly been stated in the policy, what is the timeline for reporting to the researcher and institution, so they might move to the next step, to “in collaboration with the institution or researcher, develop a risk mitigation plan” (2)? How does the timing of this review fit into the scheme of NIH study section review, funding deadlines, or other regulatory reviews?

What if there is disagreement over the elements of the risk mitigation plan or the identification of a research project as DURC? The H5N1 case illustrates how legitimate disputes about the science among experts can make true risk assessment extraordinarily difficult (9). As Bouvier stated in a recent commentary, “Where to draw the line between acceptable and unacceptable risk is a subjective assessment, made by individuals with vastly different opinions, values, backgrounds, and beliefs. We simply have no objective measure.” (10). There is no appeals process identified in the policy, nor even any point of contact at any research agency. Furthermore, elements of the risk mitigation plan, as described by the policy, such as ongoing review at the institutional level, are ill defined. For example, how often is “ongoing”? What entity or individual at the university should be tasked with “ongoing” review, and will this lead to a substantial unfunded regulatory burden at the institutional level? If this is a task given to IBCs, what additional resources will be available to support these activities? To what federal body do the IBCs report and consult? Will IBCs be establishing a relationship with the NSABB similar to that

currently shared with the Recombinant DNA Advisory Committee?

Research universities are already struggling to sustain research programs while facing increasing compliance requirements and under-recovery of indirect costs associated with federally funded research (11). Clearly, researchers or institutions are under no obligation to receive federal funding if the terms or conditions of a grant or contract are not acceptable, but this seems contrary to the nation’s public health and bio-defense needs. As the Federal Experts Security Advisory Panel recently stated in its final report,

“Discouraging researchers or institutions from federally funded research with select agents or delaying research with additional layers of review can only hurt national security.”

“work with biological select agents and toxins (BSAT) is critical to national security, appreciating that this work is important in the development and manufacturing of medical countermeasures, the ability to perform diagnostic tests, and the ability to securely transport specimens, among other activities” (12). Discouraging researchers or institutions from federally funded research with select agents or delaying research with additional layers of review can only hurt national security, the antithesis of the intent of the DURC policy.

Is the DURC Policy Inconsistent with NSDD-189 and National Security?

Originating in the Reagan Administration and reaffirmed by every subsequent administration, including that of President Obama, National Security Decision Directive (NSDD) 189 is a federal policy expressing the principle that openness is essential to scientific progress and should be preserved whenever possible (13). Although the DURC policy cites NSDD-189 specifically, the

policy itself seems to fly in the face of this directive. The risk mitigation options described in the policy include modifying research protocols, determining communications modes and venues, or requesting redaction, all of which run directly counter to NSDD-189: “No restrictions may be placed upon the conduct or reporting of federally-funded fundamental research that has not received national security classification.” Perhaps more alarming is the idea that through ongoing review, federal agencies might classify fundamental research midstream. Although technically this is an authority allowable under NSDD-189,

doing so would violate the spirit of this policy, which unequivocally states, “The strength of American science requires a research environment conducive to creativity, an environment in which the free exchange of ideas is a vital component... It is the policy of this Administration that, to the maximum extent possible, the products of fundamental research remain unrestricted.” At the very least, dual-use research has an inherent beneficial, and in the case of the life sciences often life-saving, potential: Restricting access to research results and methodology will deny researchers and the public the ability to use that research for positive societal benefit.

The feasibility of risk mitigation options related to security classification and redaction also presents challenges. The ability to retroactively or selectively control information in an extramural funding environment is, for a variety of reasons, quite limited. It was the complexity of this issue that caused the World

Health Organization (WHO) panel of experts reviewing the H5N1 papers to call for full publication of the studies, in opposition to the original NSABB recommendation (14). Public universities are subject to state open records laws that make many documents freely available to the public; research proposals, reports, and publications go through multiple reviews by many individuals and entities before publication; and much of the life sciences research covered by the policy has been going on for decades. Is it truly possible to retroactively classify research-related information that has been extraordinarily accessible for some period of time? How can you ensure that “bona fide” researchers have access to the important details of redacted publications necessary to develop medical countermeasures, without slowing research progress? Even if a system of researcher verification is established, who controls access to databases and materials? Journal publishers are private entities, and although many have acted responsibly in setting up their own

systems of DURC review short of classification, by what authority can the government or institutions prevent publication of research results?

Finally, the specter of classifying or controlling life sciences research raises larger security issues. The post-2001 expansion of biodefense research in the United States has raised questions as to whether the U.S. effort was in violation of the international BWTC (15). One of our strongest arguments against this perception is the openness of the university environment in which much of this research takes place, a transparent system that helps dispel concerns of a “secret” U.S. bio-weapons program. Classifying the research, redacting research results, or driving select agent research out of universities to national or defense laboratories will only exacerbate this negative perception of the U.S. life science research program, which could cause greater national security or diplomatic issues. We have already seen this concern play out in the H5N1 publications case, as NSABB Acting Chair Paul Keim recently testified at a Senate Homeland Security and Government Affairs Committee hearing (16).

Is There a Better Path Forward?

The life sciences and security communities represent two perspectives with one common goal of working for the public good. It is unfortunate that the controversy over the H5N1 publications has in some cases polarized what has been an evolving and productive discussion, nationally and internationally, on how to work together to foster the highest-quality, appropriately regulated biological research (1).

Reaching that goal will require balance, which in this context can perhaps be best defined as the understanding that the most stringent security measures do not necessarily translate to a commensurate increase in security, nor does scientific freedom equate to abrogation of responsibility. It has already been demonstrated that scientists, once made aware of the potential risks associated with their research, are willing to modify their research methodologies or communications to minimize the risk (17).

The proposed modification to the Select Agent Rule adds additional requirements for biosafety and biosecurity training for those personnel with any access to these dangerous pathogens (4). Although institutions conducting research with select agents or in biocontainment laboratories already typically conduct extensive training of personnel, it does not seem unreasonable to add a discussion of the risks of dual-use research as a component of that required training. There are already a number of excellent resources for DURC education available, including modules developed by the NSABB (18) and the Federation of American Scientists (5), both at the local and national level.

Finally, responsible communication of research should start with the principles set forth

by the NSABB. These include consideration of “the need for the inclusion of contextual and explanatory information that might minimize” concerns about the dangers posed by the research; an examination of the protective and oversight measures currently in place; and a full understanding and analysis of the positive benefit of the study (19).

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10.1126/science.1223995

PERSPECTIVE

Securing Medical Research: A Cybersecurity Point of View

Bruce Schneier

The problem of securing biological research data is a difficult and complicated one. Our ability to secure data on computers is not robust enough to ensure the security of existing data sets. Lessons from cryptography illustrate that neither secrecy measures, such as deleting technical details, nor national solutions, such as export controls, will work.

Science and Nature have each published papers on the H5N1 virus in humans after considerable debate about whether the research results in those papers could help terrorists create a bioweapon (1, 2). This notion of “dual use” research is an important one for the community, and one that will sooner or later become critical. Perhaps these two papers are not dangerous in the wrong hands, but eventually there will be research results that are.

My background is in cryptography and computer security. I cannot comment on the potential value or harm from any particular piece of biological research, but I can discuss what works and what does not to keep research data secure. The cryptography and computer security com-

munities have been wrestling for decades now with dual-use research: for example, whether to publish new Windows (Microsoft Corporation) vulnerabilities that can be immediately used to attack computers but whose publication helps us make the operating system more secure in the long run. From this experience, I offer five points to the virology community.

First, security based on secrecy is inherently fragile. The more secrets a system has, the less secure it is. A door lock that has a secret but unchangeable locking mechanism is less secure than a commercially purchased door lock with an easily changeable key. In cryptography, this is known as Kerckhoffs’ principle: Put all your secrecy into the key and none into the cryptographic algorithm (3, 4). The key is unique and easily changeable; the algorithm is system-wide and much more likely to become public. In fact,

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algorithms are deliberately published so that they get analyzed broadly. The lesson for dual-use virology research is that it is risky to base your security on keeping research secret. Militaries spend an enormous amount of money trying to maintain secret research laboratories, and even they do not always get security right. Once secret data become public, there is no way to go back.

Second, omitting technical details from published research is a poor security measure. We tried this in computer security with regard to vulnerabilities, announcing general information but not publishing specifics. The problem is that once the general information is announced, it is much easier for another researcher to replicate the results and generate the details. This is probably even more true in virology research than in computer security research, where the very existence of a result can provide much of the road map to that result.

Third, technical difficulty as a security measure has only short-term value. Technology only gets better; it never gets worse. To believe that some research cannot be replicated by amateurs because it requires equipment only available to state-of-the-art research institutions is short-sighted at best. What is impossible today will be a Ph.D. thesis in 20 years, and what was a Ph.D. thesis 20 years ago is a high-school science fair project today.

Fourth, securing research data in computer networks is risky at best. If you read newspapers, you know the current state of the art in computer security: Everything gets hacked. Cyber criminals steal money from banks. Cyber spies steal data from military computers. Although people talk about H5N1 research in terms of securing the research papers, that is largely a red herring; even if no papers existed, the research data would still be on a network-connected computer somewhere.

Not all computers are hacked and not all data gets stolen, but the risks are there. There are two basic types of threats in cyberspace. There are the opportunists: for example, criminals who want to break into a retail merchant's system and steal a thousand credit card numbers. Against these attackers, relative security is what matters. Because the criminals do not care whom they attack, you are safe if you are more secure than other networks. The other type of threat is a targeted attack. These are attackers who, for whatever reason, want to attack a particular network. The buzzword in Internet security for this is "advanced persistent threat" (5). It is almost impossible to secure a network against a sufficiently skilled and tenacious adversary. All we can do is make the attacker's job harder.

This does not mean that all virology data will be stolen via computer networks, but it does mean that, once the existence of that data becomes public knowledge, you should assume that the bad guys will be able to get their hands on it.

Lastly, national measures that prohibit publication will not work in an international com-

munity, especially in the Internet age. If either *Science* or *Nature* had refused to publish the H5N1 papers, they would have been published somewhere else. Even if some countries stop funding—or ban—this sort of research, it will still happen in another country.

*"This debate
will not go away;
it will only get
more urgent."*

The U.S. cryptography community saw this in the 1970s and early 1980s. At that time, the National Security Agency (NSA) controlled cryptography research, which included denying funding for research, classifying results after the fact, and using export-control laws to limit what ended up in products. This was the pre-Internet world, and it worked for a while. In the 1980s they gave up on classifying research, because an international community arose (6). The limited ability for U.S. researchers to get funding for block-cipher cryptanalysis merely moved that research to Europe and Asia. The NSA continued to limit the spread of cryptography via export-control laws; the U.S.-centric nature of the computer industry meant that this was effective. In the 1990s they gave up on controlling software because the international online community became mainstream; this period was called "the Crypto Wars" (7). Export-control laws did prevent Microsoft from embedding cryptography into Windows for over a decade, but it did nothing to prevent products made in other countries from filling the market gaps.

Today, there are no restrictions on cryptography, and many U.S. government standards are the result of public international competitions. Right now the National Institute of Standards and Technology is working on a new Secure Hash Algorithm standard (8). When it is announced next year, it will be the product of a public call for algorithms that resulted in 64 submissions from over a dozen countries and then years of international analysis. The practical effects of unrestricted research are seen in the computer security you use today: on your computer, as you browse the Internet and engage in commerce, and on your cell phone and other smart devices. Sure, the bad guys make use of this research, too, but the beneficial uses far outweigh the malicious ones.

The computer security community has also had to wrestle with these dual-use issues. In the early days of public computing, researchers who discovered vulnerabilities would quietly tell the product vendors so as to not also alert hackers. But all too often, the vendors would ignore the researchers. Because the vulnerability was not public, there was no urgency to fix it. Fixes might go into the next product release. Researchers, tired of this, started publishing the existence of vulnerabilities but not the details. Vendors, in response, tried to muzzle the researchers. They threatened them with lawsuits and belittled them in the press, calling the vulnerabilities only theoretical and not practical. The response from the researchers was predictable: They started publishing full details, and sometimes even code, demonstrating the vulnerabilities they found. This was called "full disclosure" and is the primary reason vendors now patch vulnerabilities quickly (9). Faced with published vulnerabilities that they could not pretend did not exist and that the hackers could use, they started building internal procedures to quickly issue patches. If you use Microsoft Windows, you know about "patch Tuesday," the once-a-month automatic download and installation of security patches.

Once vendors started taking security patches seriously, the research community (university researchers, security consultants, and informal hackers) moved to something called "responsible disclosure." Now it is common for researchers to alert vendors before publication, giving them a month or two head start to release a security patch. But without the threat of full disclosure, responsible disclosure would not work, and vendors would go back to ignoring security vulnerabilities (10, 11).

Could a similar process work for viruses? That is, could the makers work in concert with people who develop vaccines so that vaccines become available at the same time as the original results are released? Certainly this is not easy in practice, but perhaps it is a goal to work toward.

Limiting research, either through government classification or legal threats from vendors, has a chilling effect. Why would professors or graduate students choose cryptography or computer security if they were going to be prevented from publishing their results? Once these sorts of research slow down, the increasing ignorance hurts us all.

On the other hand, the current vibrant fields of cryptography and computer security are a direct result of our willingness to publish methods of attack. Making and breaking systems are one and the same; you cannot learn one without the other. (Some universities even offer classes in computer virus writing.) Cryptography is better, and computers and networks are more secure, because our communities openly publish details on how to attack systems.

Virology is not computer science. A biological virus is not the same as a computer virus. A

vulnerability that affects every individual copy of Windows is not as bad as a vulnerability that affects every individual person. Still, the lessons from computer security are valuable to anyone considering policies intended to encourage life-saving research in virology while at the same time prevent that research from being used to cause harm. This debate will not go away; it will only get more urgent.

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10.1126/science.1224321

POLICY FORUM

Evolution, Safety, and Highly Pathogenic Influenza Viruses

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Experience with influenza has shown that predictions of virus phenotype or fitness from nucleotide sequence are imperfect and that predicting the timing and course of evolution is extremely difficult. Such uncertainty means that the risk of experiments with mammalian-transmissible, possibly highly virulent influenza viruses remains high even if some aspects of their laboratory biology are reassuring; it also implies limitations on the ability of laboratory observations to guide interpretation of surveillance of strains in the field. Thus, we propose that future experiments with virulent pathogens whose accidental or deliberate release could lead to extensive spread in human populations should be limited by explicit risk-benefit considerations.

In response to two sets of experiments on mammalian-transmissible, modified influenza A/H5N1 viruses (1, 2) the U.S. Department of Health and Human Services has promulgated a new policy on dual-use research of concern (3). This policy, and other statements from U.S. and international bodies, identify the need for risk mitigation in future studies of mammalian-transmissible variants of highly pathogenic influenza virus and certain other infectious agents (4, 5), raising an important new question: How should funders, regulators, and researchers evaluate what future experiments should be done with such viruses? The answer to this question depends on the relative magnitude of risks and benefits of such experiments. For influenza, useful evaluations of either risks or benefits depend in part on what we know about virus evolution. Proponents of continued research in this area suggest that knowing the mutations involved in mammalian airborne transmission will aid surveillance, allowing us to see whether

there is evolution in the direction of a pandemic virus. Mitigating the risk of accidental or deliberate release of these strains, as directed by the new policy (3), also depends on how well we can predict the virulence, transmissibility, and evolutionary trajectory of influenza viruses.

We contend that predictions about how particular influenza strains will behave in humans or, even more important, how they will evolve, remain highly speculative. The most striking examples of influenza's unpredictability are in the area of drug resistance. Animal models, *in vitro* studies, and mathematical models have all contributed to our understanding of drug-resistant strains. Despite that knowledge, aspects of the spread of adamantane-resistant A/H3N2 (2003 to the present) and the spread of oseltamivir-resistant A/H1N1 (2007 to 2009) took the influenza community by surprise.

Adamantanes were used to treat influenza starting in the late 1960s. Animal models showed essentially no "fitness cost" or virulence reduction in strains with mutations conferring adamantane resistance (6), which commonly emerge during treatment (7) and can spread within families (7). These observations would have predicted a high likelihood that adamantane resistance would spread widely in populations where adamantanes were used, but such spread did not occur for decades, for reasons that remain unclear. Then, in 2003 to 2004, adamantane-resistant viruses emerged to rapidly become

the dominant influenza A/H3N2 isolates globally (8, 9). Although adamantane use likely played a role in the genesis and initial spread of the resistant lineage (8), the near-fixation of this lineage in global A/H3N2 isolates may have been due to the presence of immune escape mutations (9), a selection pressure whose importance in spreading this virus could not have been confidently predicted. Also unanticipated was the persistence of resistant viruses to the present day, despite minimal use of adamantanes (9).

Resistant strains emerge *de novo* in several percent of influenza patients treated with the neuraminidase inhibitor oseltamivir (10). However, extensive spread of resistance was not reported before 2007. At the time, this situation was explainable by the large fitness costs observed in animal models for the most common oseltamivir-resistance mutation (11). Public health officials and virologists were therefore surprised when, in winter 2007 to 2008, resistance to oseltamivir in A/H1N1 viruses carrying the H275Y mutation arose and spread widely. Even more surprising was the finding that this rise was not explained by geographic patterns of antiviral use, and high prevalence of the resistant strain was first detected in Norway, where oseltamivir use was negligible (12). Only after the global spread of this drug-resistant A/H1N1 lineage have we begun to understand the additional enabling mutations (13–15) that led to its rapid emergence. Although drug-resistant strains were obviously anticipated under drug pressure, these experiences serve as a warning against overconfidence in predicting the timing and direction of influenza evolution.

Antigenic drift of influenza A hemagglutinin is probably the best-studied evolutionary process in any virus, yet both the timing and amino acid location of changes that lead to important antigenic changes (16) in any particular strain remain difficult to predict (17). As a result, in about half of all influenza seasons, one or more components of the trivalent vaccine are poorly matched to the circulating strains of flu (18). Predictions of virus properties from sequence information are likewise imprecise, highlighting how much more needs to be learned about the effects of genetic environment on properties related to individual mutations. In the 2009

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pandemic, for example, the E627K mutation in the PB2 gene was expected to enhance virulence and promote transmission, but it did not spread widely, and it did not enhance virulence in animal models when introduced in the genetic background of the 2009 pandemic virus (19).

Contentions that H5N1 mutant strains might not be highly transmissible or as pathogenic as wild-type H5N1 strains in humans (20) may therefore be falsely reassuring. The evidence from ferrets that these viruses are transmissible between mammals by the airborne route, coupled with the demonstrated human virulence of wild-type avian A/H5N1 strains, indicate that H5N1 and its mutants should be taken seriously (21).

By emphasizing the limits of predictability, we do not mean to suggest that molecular studies of influenza transmissibility and virulence, or surveillance of animal virus populations, lack value. Viral sequences contain important information; studying the behavior of novel variants in the laboratory and adding precision to surveillance in the field are necessary if we are ever to improve our predictive powers. But because we cannot predict how these strains will behave in humans or, critically, how they will evolve, such studies on transmissible versions of dangerous zoonotic pathogens are particularly risky and deserve special treatment.

Indeed, great care was taken in the experiments now being reported (1, 2), and the World Health Organization (WHO) specifically noted that the “laboratory-modified H5N1 viruses are currently stored in well-established research facilities with high security and high safety (BSL-3+)” (5) (see Fig. 1). However, it is not a given that all future experiments will be conducted with such care, and WHO also called it “a matter of urgency” to define “the biosafety and biosecurity conditions under which further research is conducted on the laboratory-modified H5N1 viruses” (5). In doing so, we must remember that accidents happen even under high-level containment conditions. Accidental infections of laboratory workers with severe acute respiratory syndrome (SARS) coronavirus (21) and with smallpox (22), and the accidental release of foot-and-mouth disease virus from a laboratory in the United Kingdom (23), indicate the serious and real risk of laboratory accidents, a risk that increases with the number of laboratories and workers involved. The 1977 reemergence of the

A/H1N1 influenza virus after decades of absence contributed to seasonal influenza for three decades. This virus’s genetic similarity to 1950 A/H1N1 viruses suggests that it descended from a laboratory strain (24), although the exact circumstances remain unclear.

Exactly how to translate this high level of concern into the current framework of biological

has proven epidemic potential and no widely available vaccine, seems to meet all of the BSL-4 criteria yet is handled in BSL-3 laboratories. As of 2009, there were at least 14 “entities” maintaining registered BSL-4 laboratories in the United States (26), at least 24 BSL-4 laboratories worldwide (27), and at least 1495 BSL-3 laboratories registered in the United States and working with select agents (27), although the total number of BSL-3 laboratories in the United States is unknown (27). Comprehensive data from intramural laboratories at the National Institute of Allergy and Infectious Diseases estimate the risk of exposure as 2 per 100,000 operator-hours; only 1 of the 12 exposures involved in this calculation involved a clinical infection (28). Data from a larger set of U.S. laboratories indicates 26 incidents, 8 of which resulted in documented infections, in BSL-3 and BSL-4 laboratories (not including agricultural pathogens). Another five, all involving infections, are known in non-U.S. BSL-3 and BSL-4 laboratories between 2002 and 2007; in the United States and elsewhere, reporting of such incidents is incomplete (28).

The modified H5N1 strains do not fit neatly into the categories established in the biosafety guidelines: Aerosol transmissibility among humans is possible (and is a key reason for interest in their study) but unproven; vaccines may protect laboratory workers but are not available on a scale sufficient for the general population, which might be at risk if a laboratory worker were infected and were able to transmit the infection to others. Although we are not in a position to resolve the debate about the proper biosafety level, we do believe that funding agencies and regulators should limit the number of laboratories and individuals working

on these modified strains. Each additional laboratory and individual worker adds to the risk of accidental or malicious release, so there should be a strong presumption against increasing these numbers unless the benefits of an additional study are very well justified scientifically. Biological safety depends not only on the physical barriers of BSL-3 or BSL-4 facilities but also, importantly, on the experience, training, and skill of laboratory workers; these factors should also be considered when deciding who is permitted to work with these agents. At present, U.S. law requires specific safety and security



Fig. 1. Scientists working in a BSL-4 laboratory. CDC scientists connect to a supportive air hose to breathe air and to maintain positive pressure inside the protective BSL-4 lab suit.

safety is not straightforward. U.S. government guidelines (25) indicate the current highest biosafety level (BSL), BSL-4, “for work with dangerous and exotic agents that pose a high individual risk of life-threatening disease, which may be transmitted via the aerosol route and for which there is no available vaccine or therapy.” In practice, these guidelines are not followed literally: Smallpox virus, for which there are substantial vaccine stockpiles, is limited to only two BSL-4 laboratories, and Ebola virus, which transmits only by blood contact, is classified as BSL-4, whereas the SARS coronavirus, which

measures for work on select agents (including certain highly pathogenic influenza viruses). Principles for biosafety are laid out in the widely used *Biosafety in Microbiological and Biomedical Laboratories* (25). We recommend that compliance with these measures and principles be actively monitored by the Centers for Disease Control and Prevention (CDC) on a basis more frequent than the current requirement for federal inspection of BSL-3 laboratories, which is every 3 years and when a new select agent is added (27, 29). These requirements apply only within the United States; we share the concern expressed by many experts about variation in biosafety practices worldwide (27). For experiments on the evolution of viral or bacterial transmissibility to mammals, there should be an explicit requirement to justify why the research must be done with virulent strains.

Traditional peer-reviewed funding decisions evaluate scientific merit first and then undertake risk mitigation if it is considered necessary; the dual-use research of concern (DURC) policy in the United States (3) does not specify how, if at all, this approach would change. We propose that the decision about whether research on mammalian-transmissible H5N1 viruses or agents with similar potential for damage to public health should be funded or should proceed with restrictions should not be left to each department or agency, because some may lack the relevant expertise to evaluate risks and benefits in light of the overall portfolio of studies already approved or under way. A single interagency committee, including experts in fields such as evolutionary microbiology and bio-defense as well as virology, needs to review the small number of proposals identified by grant administrators or scientific review committees that involve pathogens whose accidental or deliberate release would represent a major threat to public health. In contrast to the National Science Advisory Board on Biosecurity, which is only advisory, this committee should have decision-making authority. The U.S. government is actively considering options to strengthen DURC governance, including a possible review group to provide independent assessments of research proposals. Similar considerations should motivate policies outside the United States (27).

Each additional study of mammalian-transmissible, highly pathogenic influenza will improve our understanding and may move us closer to an ability to control such viruses, but will also increase the risk of an accident that could trigger a global public health disaster, especially if evolution proceeds in an unfavorable direction. This exceptional level of risk should motivate exceptional caution by scientists, funders, and regulators worldwide.

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Acknowledgments: We thank D. Franz, F. Hayden, R. Malley, and B. Levin for helpful comments. M.L. was supported by award U54GM088558 from the National Institute of General Medical Sciences. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institute of General Medical Sciences or the National Institutes of Health. J.B.P. acknowledges funding from the Burroughs Wellcome Fund, the David and Lucile Packard Foundation, the James S. McDonnell Foundation, the Alfred P. Sloan Foundation, and grant D12AP00025 from the U.S. Department of the Interior and Defense Advanced Research Projects Agency.

10.1126/science.1223204

POLICY FORUM

Influenza: Options to Improve Pandemic Preparation

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Science and society have been struggling to find a way to protect humankind from recurring epidemics and pandemics of influenza. Here, we review the options available in the short term and also briefly address the solutions that research may make available in the long term.

Every year, seasonal influenza causes several hundred million cases and 250,000 to 500,000 deaths, of which 90 million cases and 28,000 to 111,500 deaths occur in children (1, 2). Pandemic influenza strikes periodically, infecting billions of people and potentially causing millions of deaths. Recently, two studies showing that, in the laboratory, pathogen-

ic H5N1 influenza strains can evolve to be transmissible in ferrets divided the scientific community between those saying that the studies should not have been done and/or should not be published in their entirety and those saying that the studies are useful and should be published in their entirety (3–6). Because influenza in ferrets so closely models the disease in humans, the findings of H5N1 transmissibility between ferrets suggest that transmission of such strains between humans also could occur. The controversy continues, and it is evident that the studies have reminded us that a deadly H5N1 pandemic is not impossible. Therefore, it is important to discuss what options

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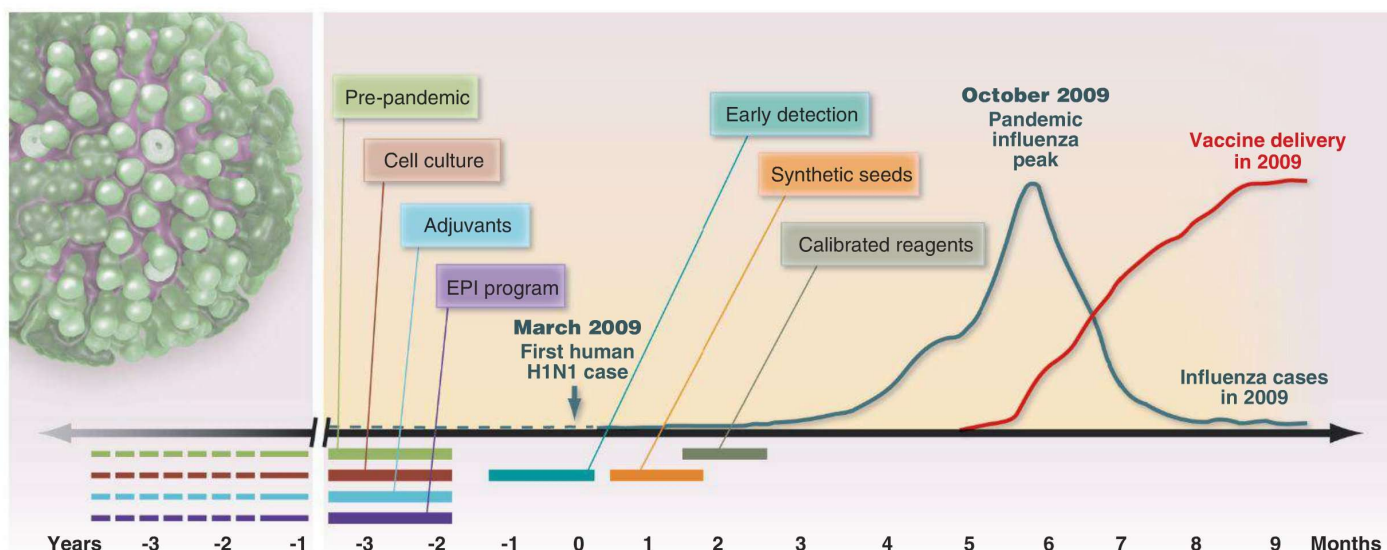


Fig. 1. Summary of the activities that occurred in 2009 from day 0 of the pandemic on 18 March 2009, when the first case of H1N1 virus was reported in Mexico, to November and December (months 8 and 9), when large quantities of the pandemic vaccine became available. Data are adapted from (22, 23); only qualitative data are shown. The figure shows that in 2009 the

vaccine became available after the peak of viral infection. The options available today to improve pandemic preparation are shown above the graph. The four options on the left are preparations in advance of a pandemic; the three on the right are elements of executing a pandemic response. [Source: Doug Jordan/CDC]

are available and whether we are ready to invest to reduce or eliminate the risk.

The experience of the 2009 H1N1 pandemic showed that pandemic vaccines could be successfully developed and deployed; however, they became available in significant quantities only after the peak of viral infection and only in high-income countries (Fig. 1). The figure also shows a summary of the activities that occurred during the 8 months between March 18, when the first case was reported in Mexico, and November, when the vaccine started to be distributed in large quantities. In the figure, we list options for improved pandemic responses.

The first option is prepandemic vaccination, which can circumvent the daunting logistics of developing, manufacturing, and distributing a new vaccine more rapidly than an advancing pandemic wave can spread. Vaccination of human subjects with an H5 vaccine (combined with an enhancer of immune responses known as an adjuvant) elicits protective antibody titers in the short term and primes the immune system for the long term against any H5 virus variant (7). The experience of 2009 indicates that, once the immune system is primed, the risk of a severe pandemic is eliminated, and the worst that can happen is a mild pandemic. During the 2009 H1N1 pandemic, most people had been primed by infection and/or vaccination with distantly related H1N1 antigens and were spared severe disease (8–10). Because H5N1 infection of humans is so rare, the population has not been primed by previous exposures. This lack of prior exposure makes the population more vulnerable to severe

disease caused by highly virulent H5N1 strains and makes immunization more difficult, necessitating the use of adjuvants, higher antigen content, and two doses to elicit sufficient antibody titers (7).

Given that licensed H5N1 vaccines are available, we have the option to vaccinate individuals at greatest risk or to vaccinate more broadly, including the populations of individual countries, of continents, or even of the entire globe. It is just a question of evaluating the cost, the logistics, and the risk of implementing such a large vaccination campaign. It is not impossible. With sufficient investment and political will, the global population could probably be vaccinated with one or two doses of a prepandemic vaccine in a period of 3 to 5 years, which would dramatically reduce the risk of a devastating H5N1 influenza pandemic.

The second option is increasing manufacturing capacity so that we can produce enough vaccines to protect the 7 billion people on our planet. The proportion of the population that must be immunized to quell a pandemic through herd immunity varies with the transmissibility of the strain, the level of preexisting immunity, and the efficacy of available vaccines. With a highly transmissible strain (such as the 1918 pandemic strain), in a population with little preexisting immunity, and using vaccines that are incompletely effective, modeling indicates that the proportion of the population that must be immunized for effective immunity is likely to be greater than 80% (11). The introduction of cell culture technologies can eliminate the current dependence on egg supply,

potentially shorten production time, and increase capacity. Although most of the vaccine doses distributed in the 2009 pandemic response were produced in eggs, a vaccine produced in cultured mammalian cells was at the leading edge of the vaccine supply (12). Cell culture production is more rapidly scalable than egg production and is not vulnerable to decimation of poultry flocks by a pandemic avian influenza strain. In the medium term, production of influenza vaccine antigens from recombinant platforms, such as *Escherichia coli*, insect cells, or plants, also could increase the global vaccine supply (13). However, in the present environment, capacity would be available only for rich countries, where it is sustained by the seasonal influenza business. Although, during the 2009 pandemic, the calculated capacity may have reached a peak of ~900 million doses, to be sustainable, capacity must be supported by the seasonal influenza business. As of 2009, the northern hemisphere seasonal influenza vaccine markets supported production of ~470 million doses of trivalent influenza vaccine (targeting three varieties of influenza) during a 6-month northern hemisphere manufacturing campaign, which corresponds to 1.4 billion doses of monovalent vaccine (14). The capacity to make sufficient bulk vaccine could be doubled or even quadrupled by the use of an adjuvant, on the basis of the reduced antigen dose needed in influenza vaccines with adjuvant (12). Reduced bulk vaccine requirements with dose-sparing adjuvants could make the capacity to fill and distribute vaccine doses a limiting factor. Therefore, start-to-finish manufacturing capacity is still too little by far compared with the

need of 7 billion doses in 6 months to vaccinate the global population during a pandemic (such as the 2009 H1N1 pandemic) in which one dose of vaccine is required or 14 billion doses in 6 months for a pandemic (such as a highly pathogenic H5N1 pandemic) in which two doses are required.

A win-win solution to the problem of insufficient influenza vaccine manufacturing capacity is possible. Today, influenza vaccines are used only in rich countries, and outside of the United States, routine influenza immunization is usually not recommended for children and infants. Recently, it has been shown that influenza vaccines with adjuvant are highly efficacious in infants and prevent 86% of the infections (15). Introducing influenza vaccines in the Extended Program on Immunization (EPI) and vaccinating all children and pregnant women globally against influenza (16) could save the lives of up to 28,000 to 111,500 infants annually, prevent 90 million cases, and enable a sustainable increase in vaccine manufacturing capacity to levels close to those required for an effective global pandemic response. Because much of the new capacity would be built in low-income countries, the expansion would end the embarrassing and inequitable situation in which influenza vaccines are only available for rich countries, as occurred during the 2009 H1N1 pandemic.

The next option is acceleration of vaccine manufacturing during a pandemic. In 2009, it took nearly 3 months from March 18, when the first case occurred, to June 7, when vaccine manufacturing started. This could probably be reduced to a couple of weeks by early detection of the first case and the use of fully synthetic seed viruses for vaccine production. Today, once the sequence of the virus is available, we can synthesize the genes and make a synthetic virus to seed vaccine manufacture in less than a week. To implement synthetic seed generation in influenza vaccine manufacturing, several changes are necessary, including a rethinking of the regulations and more rapid and widespread sharing of sequence and antigenic information about new influenza strains. Preoptimized and preapproved influenza virus backbones (sets of influenza gene segments other than those that encode the strain-specific antigens) can be designed for high yields in cell culture and eggs, as well as for attenuation, thereby increasing the vaccine supply while allowing manufacture in biosafety level 2 containment. The ability of manufacturers to synthesize vaccine seed viruses would allow them to begin developing a pandemic vaccine at their own risk, at the first hint of a potential pandemic, rather than waiting for a vaccine virus to arrive in the mail from a World Health Association (WHO)-associated laboratory. Early development activities for potential pandemic vaccines can occur on a rolling basis, even as seasonal vaccine production continues. The combination of early

detection of new strains with pandemic potential, more rapid and open sharing of surveillance data (including the results of antigenic testing), and synthetic vaccine seeds could hasten the start of pandemic vaccine manufacturing by months. Increased yields from higher-producing seed viruses could hasten the ramp-up of the vaccine supply and improve global access to pandemic vaccines.

In 2009, the distribution by regulatory authorities of the calibrated reagents required to formulate and release the pandemic vaccine was a rate-limiting step, with clinical trials starting on the basis of manufacturers' surrogate assays (12). To accelerate pandemic response and to realize the benefits of more-rapid vaccine seed virus generation, we need to eliminate the need for calibrated reagents distributed by regulatory agencies. Today, these reagents are obtained by immunizing sheep with research-grade vaccine antigen, and they are used to determine the quantity of influenza hemagglutinin (HA) in vaccines, using single radial immunodiffusion (SRID), an assay that is more than 35 years old (17). Producing and calibrating SRID reagents requires nearly 2 months. Today, there are numerous techniques (including reversed-phase high-pressure liquid chromatography and isotope dilution mass spectrometry) that can quantify vaccine antigen content with better precision than SRID (18, 19). If coupled to a simple physical technique to separate misfolded from native HA, the assay adjustments needed to formulate and release a new pandemic vaccine could be available in hours rather than months. These two technological innovations, synthetic vaccine seeds and physical release assays, could transform the WHO system for pandemic response from a mid-20th-century system of producing and distributing materials from centralized laboratories at a pace dictated by sheep seroconversion, manipulation of influenza viruses in chicken eggs, and shipping logistics, into a 21st-century system of instantaneous electronic information exchange followed by immediate production at manufacturing sites around the globe. If the above changes had been introduced before the 2009 pandemic, the vaccine would have been available in large quantities before the peak of viral infection.

Finally, in the long term, we may think of universal vaccines (13). Although these are not an immediate solution, recent findings on the molecular mechanisms of human immunity to influenza have shown that antibodies directed against the stem of influenza HA can be broadly neutralizing (20). This finding raised the expectation that, by studying these antibodies and their crystal structures in complex with HA, we may learn how to make universal vaccines. However, to date, the progress in designing universal immunogens by using conserved regions of virulence factors has been limited, which suggests that, although this is a possibility in the long term, in

the short term we should not rely only on this approach. A promising way to improve the quantity of antibodies against the conserved region of the stem seems to be a prime-boost regime, in which a DNA vaccine primes and a conventional vaccine boosts the immune response (21). This approach, possibly coupled with the use of adjuvants, has the potential to improve cross-protection by influenza vaccines in the medium term, although adjuvants and priming alone are unlikely to produce a truly universal vaccine. Finally, there are many other approaches to universal vaccines, including the use of conserved antigens that do not elicit neutralizing antibodies, such as parts of the M1, M2, and NP proteins (13). Nevertheless, these approaches have been in the research-and-development pipeline with little progress for more than two decades, and it would be surprising if they succeeded in the near term.

In conclusion, while we wait for the development of a universal influenza vaccine, we have practical options that we could implement today to reduce the risk of mass global mortality from the next influenza pandemic.

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10.1126/science.1221466

Airborne Transmission of Influenza A/H5N1 Virus Between Ferrets

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Highly pathogenic avian influenza A/H5N1 virus can cause morbidity and mortality in humans but thus far has not acquired the ability to be transmitted by aerosol or respiratory droplet ("airborne transmission") between humans. To address the concern that the virus could acquire this ability under natural conditions, we genetically modified A/H5N1 virus by site-directed mutagenesis and subsequent serial passage in ferrets. The genetically modified A/H5N1 virus acquired mutations during passage in ferrets, ultimately becoming airborne transmissible in ferrets. None of the recipient ferrets died after airborne infection with the mutant A/H5N1 viruses. Four amino acid substitutions in the host receptor-binding protein hemagglutinin, and one in the polymerase complex protein basic polymerase 2, were consistently present in airborne-transmitted viruses. The transmissible viruses were sensitive to the antiviral drug oseltamivir and reacted well with antisera raised against H5 influenza vaccine strains. Thus, avian A/H5N1 influenza viruses can acquire the capacity for airborne transmission between mammals without recombination in an intermediate host and therefore constitute a risk for human pandemic influenza.

Influenza A viruses have been isolated from many host species, including humans, pigs, horses, dogs, marine mammals, and a wide range of domestic birds, yet wild birds in the orders Anseriformes (ducks, geese, and swans) and Charad-

riiformes (gulls, terns, and waders) are thought to form the virus reservoir in nature (*1*). Influenza A viruses belong to the family Orthomyxoviridae; these viruses have an RNA genome consisting of eight gene segments (*2, 3*). Segments 1 to 3 encode the polymerase proteins: basic polymerase 2 (PB2), basic polymerase 1 (PB1), and acidic polymerase (PA), respectively. These proteins form the RNA-dependent RNA polymerase complex responsible for transcription and replication of the viral genome. Segment 2 also encodes a

second small protein, PB1-F2, which has been implicated in the induction of cell death (*4, 5*). Segments 4 and 6 encode the viral surface glycoproteins hemagglutinin (HA) and neuraminidase (NA), respectively. HA is responsible for binding to sialic acids (SAs), the viral receptors on host cells, and for fusion of the viral and host cell membranes upon endocytosis. NA is a sialidase, responsible for cleaving SAs from host cells and virus particles. Segment 5 codes for the nucleocapsid protein (NP) that binds to viral RNA and, together with the polymerase proteins, forms the ribonucleoprotein complexes (RNPs). Segment 7 codes for the viral matrix structural protein M1 and the ion-channel protein M2 that is incorporated in the viral membrane. Segment 8 encodes the nonstructural protein NS1 and the nucleic-export protein (NEP) previously known as NS2. NS1 is an antagonist of host innate immune responses and interferes with host gene expression, whereas NEP is involved in the nuclear export of RNPs into the cytoplasm before virus assembly (*2, 3*).

Influenza A viruses show pronounced genetic variation of the surface glycoproteins HA and NA (*1*). Consequently, the viruses are classified based on the antigenic variation of the HA and NA proteins. To date, 16 major antigenic variants of HA and 9 of NA have been recognized in wild birds and are found in numerous combinations designated as virus subtypes (for instance, H1N1, H5N1, H7N7, and H16N3), which are used in influenza A virus classification and nomenclature (*1, 6*). This classification system is biologically relevant, as natural host antibodies that recognize

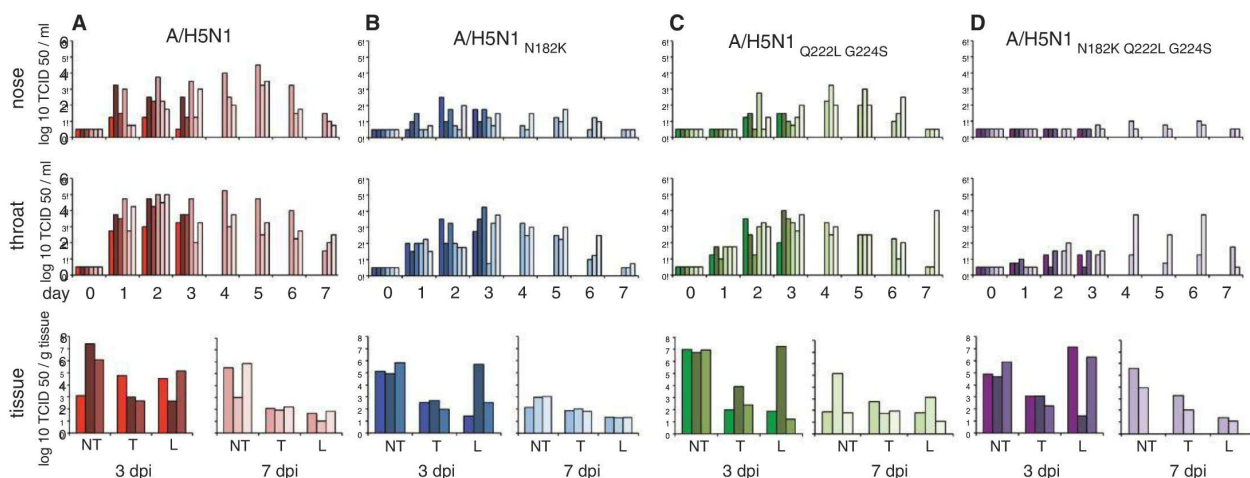


Fig. 1. In experiment 1, we inoculated groups of six ferrets intranasally with 1×10^6 TCID₅₀ of (A) influenza A/H5N1_{wildtype} virus and the three mutants (B) A/H5N1_{HA N182K}, (C) A/H5N1_{HA Q222L, G224S}, and (D) A/H5N1_{HA N182K, Q222L, G224S}. Three animals were euthanized at day 3 for tissue sampling and at day 7, when this experiment was stopped. Virus titers were measured daily in nose swabs (top) and throat swabs (middle) and also on 3 and 7 dpi in respiratory tract tissues (bottom) of individual ferrets. Virus titers in swabs and nasal turbinates (NT), trachea (T), and lungs (L) were determined by end-point titration in MDCK cells. [One animal inoculated with A/H5N1_{HA N182K, Q222L, G224S} died at 1 dpi due to circumstances not related to the experiment (D).] (Top two rows) Virus

shedding from the URT as determined by virus titers in nasal and throat swabs was highest in A/H5N1_{wildtype}-inoculated animals. The mutant that yielded the highest virus titers during the 7-day period was A/H5N1_{HA Q222L, G224S}, but titers were ~1 log lower than for the A/H5N1_{wildtype}-inoculated animals. In the first 3 days, when six animals per group were present, no significant differences were observed between A/H5N1_{HA N182K}- and A/H5N1_{HA Q222L, G224S}-inoculated animals, as calculated by comparing the viral titer (Mann-Whitney test, $P = 0.589$ and 0.818 for nose and throat titers, respectively). (Bottom row) No marked differences in virus titers in respiratory tissues were observed between the four groups. Each bar color denotes a single animal.

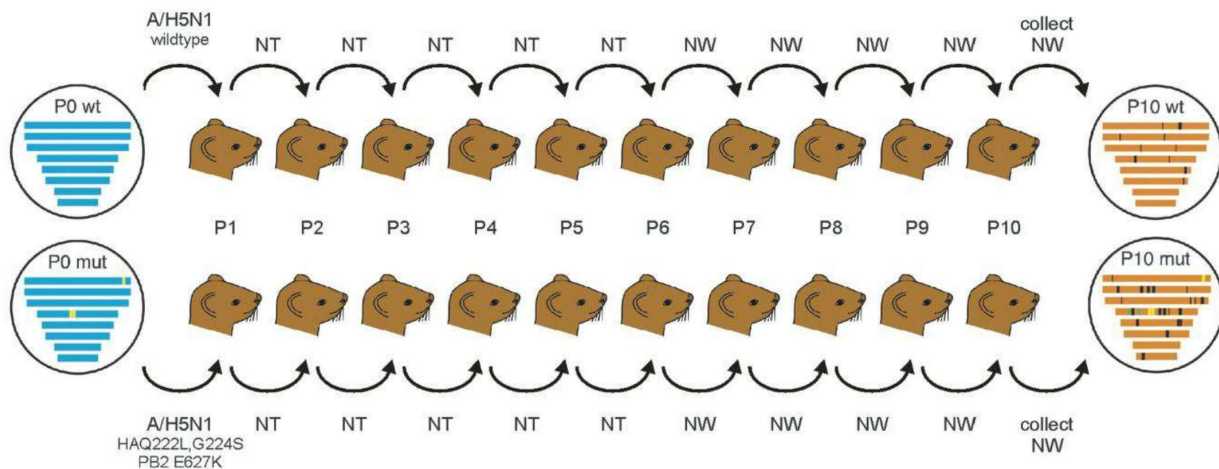


Fig. 2. Experiment 3, virus passing in ferrets (P1 to P10, passages 1 to 10). Because no airborne transmission was observed in experiment 2, A/H5N1_{wildtype} and A/H5N1_{HA Q222L,G224S PB2 E627K} were serially passed in ferrets to allow adaptation for efficient replication in mammals. Each virus was inoculated intranasally with 1×10^6 TCID₅₀ in one ferret ($2 \times 250 \mu\text{l}$, divided over both nostrils). Nose and throat swabs were collected daily. Animals were euthanized at 4 dpi, and nasal turbinates and lungs were collected. Nasal turbinates were homogenized in virus-transport medium, and this homogenate was used to inoculate the next ferret, resulting in passage 2 (fig. S6). Subsequent passages

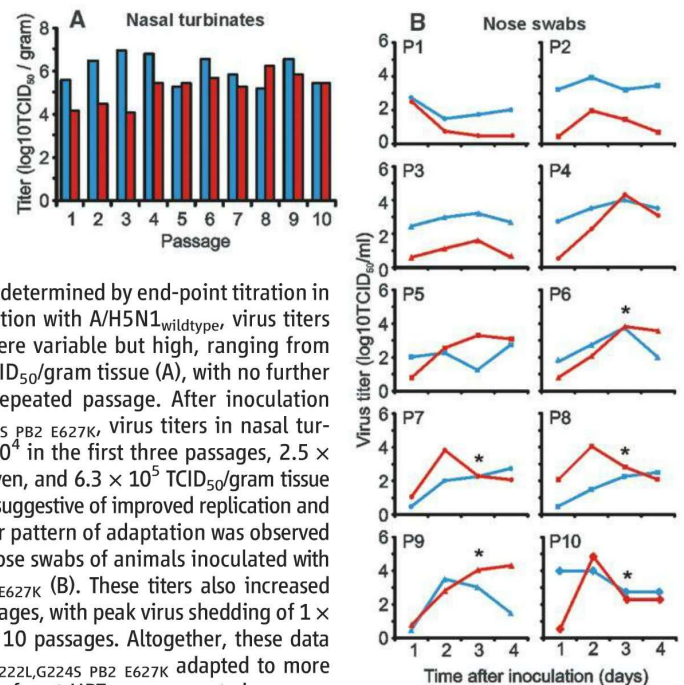
3 to 6 were performed in the same way. From passage six onward, nasal washes (NW) were collected at 3 dpi in addition to the nasal swabs. To this end, 1 ml of PBS was delivered drop wise into the nostrils of the ferrets, thereby inducing sneezing. Approximately 200 μl of the sneeze was collected in a Petri dish, and PBS was added to a final volume of 2 ml. For passages 7 through 10, the nasal-wash sample was used for the passages in ferrets. The passage-10 nasal washes were subsequently used for sequence analyses and transmission experiments to be described in experiment 4. For details, see the supplementary materials.

one HA or NA subtype will generally not cross-react with other HA and NA subtypes.

On the basis of their virulence in chickens, influenza A viruses of the H5 and H7 subtypes can be further classified into highly pathogenic avian influenza (HPAI) and low-pathogenic avian influenza (LPAI) viruses. Viruses of subtypes H1 to H4, H6, and H8 to H16 are LPAI viruses. The vast majority of H5 and H7 influenza A viruses are also of the LPAI phenotype. HPAI viruses are generally thought to arise in poultry after domestic birds become infected by LPAI H5 and H7 viruses from the wild-bird reservoir (7, 8). The HA protein of influenza A viruses is initially synthesized as a single polypeptide precursor (HA0), which is cleaved into HA1 and HA2 subunits by trypsin-like proteases in the host cell. The switch from LPAI to HPAI virus phenotype occurs upon the introduction of basic amino acid residues into the HA0 cleavage site, also known as the multibasic cleavage site (MBCS). The MBCS in HA can be cleaved by ubiquitously expressed host proteases; this cleavage facilitates systemic virus replication and results in mortality of up to 100% in poultry (9, 10).

Since the late 1990s, HPAI A/H5N1 viruses have devastated the poultry industry of numerous countries in the Eastern Hemisphere. To date, A/H5N1 has spread from Asia to Europe, Africa, and the Middle East, resulting in the death of hundreds of millions of domestic birds. In Hong Kong in 1997, the first human deaths directly attributable to avian A/H5N1 virus were recorded (11). Since 2003, more than 600 laboratory-confirmed cases of HPAI A/H5N1 virus infections in humans have been reported from 15 countries

Fig. 3. Virus titers in (A) the nasal turbinates collected at day 4 and (B) nose swabs collected daily until day 4, from ferrets inoculated with A/H5N1_{wildtype} (blue) and A/H5N1_{HA Q222L,G224S PB2 E627K} (red) throughout the 10 serial passages described in Fig. 2. Virus titers were determined by end-point titration in MDCK cells. After inoculation with A/H5N1_{wildtype}, virus titers in the nasal turbinates were variable but high, ranging from 1.6×10^5 to 7.9×10^6 TCID₅₀/gram tissue (A), with no further increase observed with repeated passage. After inoculation with A/H5N1_{HA Q222L,G224S PB2 E627K}, virus titers in nasal turbinates averaged 1.6×10^4 in the first three passages, 2.5×10^5 in passages four to seven, and 6.3×10^5 TCID₅₀/gram tissue in the last three passages, suggestive of improved replication and virus adaptation. A similar pattern of adaptation was observed in the virus titers in the nose swabs of animals inoculated with A/H5N1_{HA Q222L,G224S PB2 E627K} (B). These titers also increased during the successive passages, with peak virus shedding of 1×10^5 TCID₅₀ at 2 dpi after 10 passages. Altogether, these data indicate that A/H5N1_{HA Q222L,G224S PB2 E627K} adapted to more efficient replication in the ferret URT upon repeated passage, with evidence for such adaptation by passage number 4. In contrast, analyses of the virus titers in the nose swabs of the ferrets collected at 1 to 4 dpi throughout the 10 serial passages with A/H5N1_{wildtype} revealed no changes in patterns of virus shedding. Asterisks indicate that a nose wash was collected before the nose swab was taken, which may influence the virus titer that was detected.



(12). Although limited A/H5N1 virus transmission between persons in close contact has been reported, sustained human-to-human transmission of HPAI A/H5N1 virus has not been detected (13–15). Whether this virus may acquire the ability to be transmitted via aerosols or respiratory

droplets among mammals, including humans, to trigger a future pandemic is a key question for pandemic preparedness. Although our knowledge of viral traits necessary for host switching and virulence has increased substantially in recent years (16, 17), the factors that determine airborne

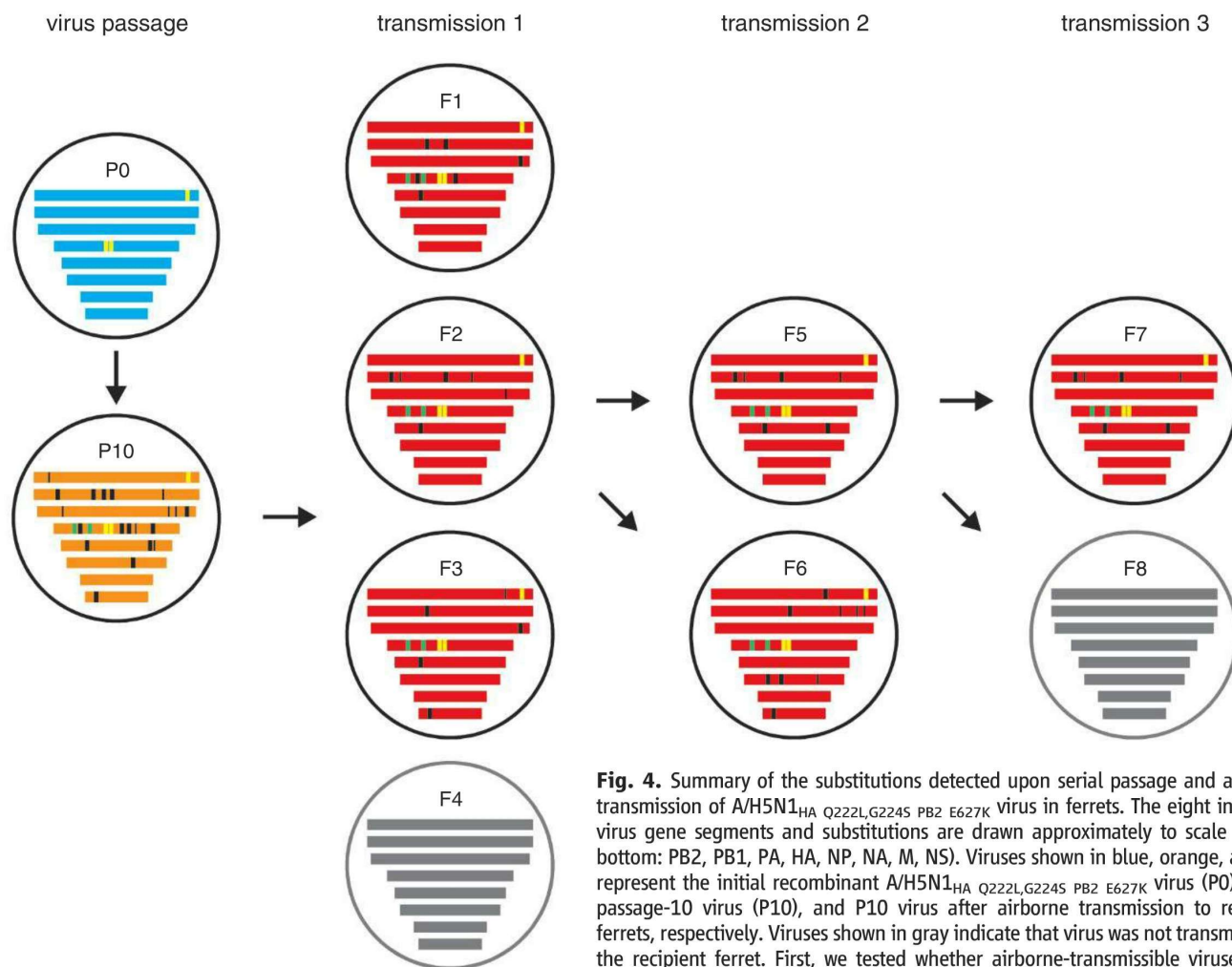


Fig. 4. Summary of the substitutions detected upon serial passage and airborne transmission of A/H5N1_{HA Q222L,G224S PB2 E627K} virus in ferrets. The eight influenza virus gene segments and substitutions are drawn approximately to scale (top to bottom: PB2, PB1, PA, HA, NP, NA, M, NS). Viruses shown in blue, orange, and red represent the initial recombinant A/H5N1_{HA Q222L,G224S PB2 E627K} virus (P0), ferret passage-10 virus (P10), and P10 virus after airborne transmission to recipient ferrets, respectively. Viruses shown in gray indicate that virus was not transmitted to the recipient ferret. First, we tested whether airborne-transmissible viruses were present in the heterogeneous virus population of ferret P10. We inoculated four

donor ferrets intranasally, which were then housed in transmission cages and paired with four recipient ferrets. Transmissible viruses were isolated from three out of four recipient ferrets (F1 to F3). Next, we took a throat-swab sample from F2 (this sample contained the highest virus titer among the positive recipient ferrets), and this sample was used to inoculate two more donor ferrets intranasally. In a transmission experiment, these donors infected two recipient ferrets via airborne transmission (F5 and F6). Virus isolated from F5 was passaged once in MDCK cells and was subsequently used in a third transmission experiment in which two intranasally inoculated donor ferrets transmitted the virus to one of two recipient ferrets (F7). The genetic composition of the viral quasi-species present in the nasal wash of ferret P10 was determined by sequence analysis using the 454/Roche GS-FLX sequencing platform. Conventional Sanger sequencing was used to determine the consensus sequence in one high-titer nasal- or throat-swab sample for each ferret. Thick and thin vertical bars indicate amino acid and nucleotide substitutions, respectively; substitutions introduced by reverse genetics are shown in yellow; substitutions detected in passage 10 and all subsequent transmissions are shown in green.

transmission of influenza viruses among mammals, a trait necessary for a virus to become pandemic, have remained largely unknown (18–21). Therefore, investigations of routes of influenza virus transmission between animals and on the determinants of airborne transmission are high on the influenza research agenda.

The viruses that caused the major pandemics of the past century emerged upon reassortment (that is, genetic mixing) of animal and human influenza viruses (22). However, given that viruses from only four pandemics are available for analyses, we cannot exclude the possibility that a future pandemic may be triggered by a wholly avian virus without the requirement of reassortment. Several studies have shown that reassortment

events between A/H5N1 and seasonal human influenza viruses do not yield viruses that are readily transmitted between ferrets (18–20, 23). In our work, we investigated whether A/H5N1 virus could change its transmissibility characteristics without any requirement for reassortment.

We chose influenza virus A/Indonesia/5/2005 for our study because the incidence of human A/H5N1 virus infections and fatalities in Indonesia remains fairly high (12), and there are concerns that this virus could acquire molecular characteristics that would allow it to become more readily transmissible between humans and initiate a pandemic. Because no reassortants between A/H5N1 viruses and seasonal or pandemic

human influenza viruses have been detected in nature and because our goal was to understand the biological properties needed for an influenza virus to become airborne transmissible in mammals, we decided to use the complete A/Indonesia/5/2005 virus that was isolated from a human case of HPAI A/H5N1 infection.

We chose the ferret (*Mustela putorius furo*) as the animal model for our studies. Ferrets have been used in influenza research since 1933 because they are susceptible to infection with human and avian influenza viruses (24). After infection with human influenza A virus, ferrets develop respiratory disease and lung pathology similar to that observed in humans. Ferrets can also transmit human influenza viruses to other ferrets that

serve as sentinels with or without direct contact (fig. S1) (25–27).

Host restriction of replication and transmission of influenza A viruses is partly determined by specific SA receptors on the surface of susceptible cells. The affinity of influenza viruses for these receptors varies according to the species from which they are isolated. Influenza viruses of avian origin preferentially bind to α -2,3-linked SA receptors, whereas human influenza viruses recognize α -2,6-linked SA receptors. The receptor distribution in ferrets resembles that of humans in that the α -2,6-linked SA receptors are predominantly present in the upper respiratory tract (URT), and the α -2,3-linked SA receptors are mainly present in the lower respiratory tract. In chickens and other birds, α -2,3-linked SAs predominate, but both α -2,3-linked and α -2,6-linked SA are present throughout the respiratory and enteric tracts (fig. S2) (28). The differences in receptor distribution between humans and avian species are thought to determine the host restriction of influenza A viruses. A switch in receptor specificity from avian α -2,3-SA to human α -2,6-SA receptors, which can be acquired by specific mutations in the receptor binding site (RBS) of the HA, is expected to be necessary for an avian virus to become transmissible and, thus, gain the potential to become pandemic in humans.

Besides a switch in receptor specificity to facilitate infection of cells in the URT, increased virus production in the URT and efficient release of virus particles from the respiratory tract to yield airborne virus may also be required (22). Such traits are likely to be determined by the viral surface glycoproteins and the proteins that form the viral polymerase complex. Amino acid substitutions in the polymerase proteins have already been shown to be major determinants of host range and transmission, including for pandemic influenza viruses (29–31). Whereas avian viruses, in principle, replicate at temperatures around 41°C (the temperature in the intestinal tract of birds), for replication in humans the viruses need to adapt to 33°C (the temperature of the human URT). The amino acid substitution Glu⁶²⁷→Lys⁶²⁷ (E627K) in the polymerase complex protein PB2 has been associated with increased virus replication in mammalian cells at such lower temperatures (16, 17, 32).

In addition, when newly formed virus particles bud from the host cell membrane after virus replication, the NA present on the virus membrane facilitates the release of particles. For A/H5N1, this process is rather inefficient, and released particles tend to form virus aggregates (22). Therefore, a balance between the properties endowed by HA and NA may be required to generate single particles. These established effects were thus used as the basis for the initial substitutions chosen in the current study.

Fig. 5. Airborne transmission of A/H5N1 viruses in ferrets. Transmission experiments are shown for A/H5N1_{wildtype} (A and B) and A/H5N1_{HA Q222L,G224S PB2 E627K} (C and D) after 10 passages (P10) in ferrets. Two or four ferrets were inoculated intranasally with nasal-wash samples collected from P10 virus of A/H5N1_{wildtype} and A/H5N1_{HA Q222L,G224S PB2 E627K}, respectively, and housed individually in transmission cages (A and C). A naïve recipient ferret was added to each transmission cage adjacent to a donor ferret at 1 dpi (B and D). Virus titers in throat (black bars) and nose swabs (white bars) were determined by end-point titration in MDCK cells. Geometric mean titers and SDs (error bars) of positive samples are shown. The number of animals infected via airborne transmission is indicated in (D) for each time point after exposure; the drop from three animals infected at day 7 to one animal at day 9 and no animals at day 11 is explained by the fact that the animals that became infected via airborne transmission had cleared the virus by the end of the experiment and, therefore, detectable amounts of virus were no longer present. The dotted lines indicate the lower limit of virus detection.

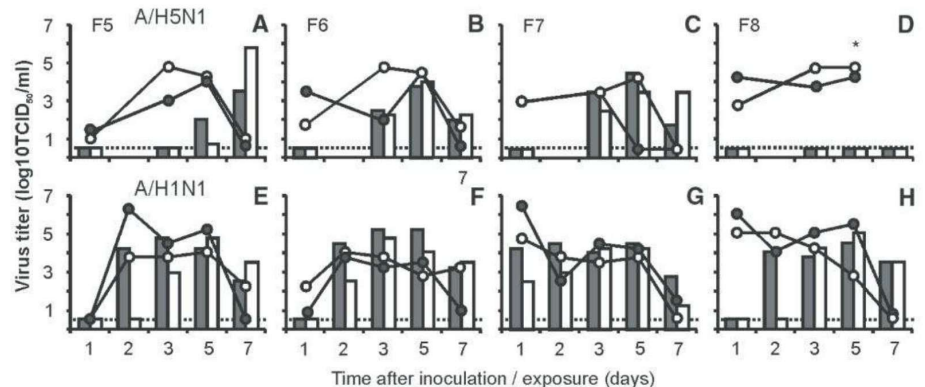
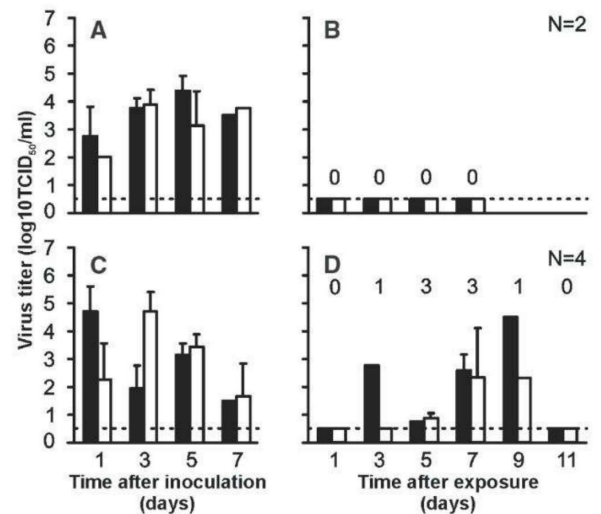


Fig. 6. Comparison of airborne transmission of experimental passaged A/H5N1 and 2009 pandemic A/H1N1 viruses in individual ferrets. A throat-swab sample from ferret F2 at 7 days postexposure (dpe) (Fig. 5D) was used for the transmission experiments shown in (A) and (B), and a virus isolate obtained from a nose swab collected from ferret F5 at 7 dpi (Fig. 6A) was used for the experiments in (C) and (D). For comparison, published data on transmission of 2009 pandemic A/H1N1 virus between ferrets is shown in (E) to (H) (27). Data for individual transmission experiments is shown in each panel, with virus shedding in inoculated and airborne virus-exposed animals shown as lines and bars, respectively. For the transmission experiments with airborne-transmissible A/H5N1 (A to D), nose or throat swabs were not collected at 2 dpi and 2 dpe. White circles and bars represent shedding from the nose; black circles and bars represent shedding from the throat. The asterisk indicates the inoculated animal that died 6 days after intranasal inoculation.

Human-to-human transmission of influenza viruses can occur through direct contact, indirect contact via fomites (contaminated environmental surfaces), and/or airborne transmission via small aerosols or large respiratory droplets. The pandemic and epidemic influenza viruses that have circulated in humans throughout the past century were all transmitted via the airborne route, in contrast to many other respiratory viruses that are exclusively transmitted via contact. There is no exact particle size cut-off at which transmission changes from exclusively large droplets to aro-

sols. However, it is generally accepted that for infectious particles with a diameter of 5 μ m or less, transmission occurs via aerosols. Because we did not measure particle size during our experiments, we will use the term “airborne transmission” throughout this Report.

Biosafety and biosecurity concerns have remained foremost in our planning for this research program. The details are explained in the supplementary materials and are summarized here: The enhanced Animal Biosafety Laboratory level 3 (ABSL3+) facility at Erasmus Medical Center

Table 1. Lethality of WT and airborne-transmissible A/H5N1 virus in ferrets upon inoculation via different routes. *n*, number of animals; N.A., not applicable.

Inoculation route	Virus	Dead or moribund (no. dead/no. tested)	Day of death postinoculation (no.)
Intratracheal	A/H5N1 _{wildtype}	6/6*	2 (<i>n</i> = 2), 3 (<i>n</i> = 4)
	A/H5N1/F5	6/6	3 (<i>n</i> = 6)
Intranasal	A/H5N1 _{wildtype} /P10	2/2†	6 (<i>n</i> = 2)
	A/H5N1 _{HA} Q222L,G224S PB2 E627K/P10	0/4	N.A.
	A/H5N1/F2	0/2	N.A.
	A/H5N1/F5	1/2	6 (<i>n</i> = 1)
Airborne	A/H5N1 _{wildtype}	N.A.	N.A.
	A/H5N1 _{HA} Q222L,G224S PB2 E627K/P10	0/3	N.A.
	A/H5N1/F2	0/2	N.A.
	A/H5N1/F5	0/1	N.A.

*These data refer to a published study (45). †These ferrets were inoculated with P10 H5N1_{wildtype} virus, but data are consistent with previous studies that used larger groups of animals inoculated with the original strain (39, 40).

Table 2. Receptor specificity of the different mutant A/H5N1 viruses, as determined by a modified TRBC hemagglutination assay. Introduction of Q222L and G224S in the A/H5N1 HA resulted in a receptor binding preference switch from the avian α -2,3- to the human α -2,6-linked SA receptor. Subsequent substitution of H103Y and T156A resulted in an increased affinity for α -2,3- and α -2,6-linked SA, in agreement with glycan array studies (51). For details, see supplementary experiment 9. HAU, hemagglutination units.

Virus	Subtype	HA titer (HAU/50 μ l)		
		TRBC	α -2,3-linked TRBC	α -2,6-linked TRBC
A/Netherlands/213/03	H3N2	64	0	64
A/Vietnam/1194/04	H5N1	64	64	0
A/H5N1 _{PB2 E627K}	H5N1	64	16	0
A/H5N1 _{HA H103Y,T156A PB2 E627K}	H5N1	64	48	0
A/H5N1 _{HA Q222L,G224S PB2 E627K}	H5N1	64	0	24
A/H5N1 _{HA H103Y,T156A,Q222L,G224S PB2 E627K}	H5N1	64	4	32

(MC) Rotterdam, the Netherlands, was constructed for the specific purpose of containing pathogenic and transmissible influenza viruses and other pathogens of concern. The facility consists of a negatively pressurized laboratory with an interlock room. All in vivo and in vitro experimental work is carried out in negatively pressurized class 3 isolators or class 3 biosafety cabinets, respectively. The facility is secured by procedures recognized as appropriate by the institutional biosafety officers and facility management at Erasmus MC, as well as Dutch and U.S. government inspectors.

Before and during the research, biosafety officers of Erasmus MC and inspectors from the Dutch government, as well as from the U.S. Centers for Disease Control and Prevention, approved the facilities and procedures. Explicit permits for research on genetically modified airborne-transmissible A/H5N1 virus were obtained from the Dutch government. The research was performed strictly in accordance with the Dutch Code of Conduct for Biosecurity (33). All personnel were instructed and trained extensively for working in the ABSL3+ facility, handling (highly pathogenic) influenza virus, and controlling incidents (such as spills). To further prevent occupational risks, research personnel used protective equipment and were offered seasonal

and A/H5N1 influenza vaccines (25). For emergency purposes, Erasmus MC holds supplies of oseltamivir and has quarantine hospital rooms.

Using a combination of targeted mutagenesis followed by serial virus passage in ferrets, we investigated whether A/H5N1 virus can acquire mutations that would increase the risk of mammalian transmission (34). We have previously shown that several amino acid substitutions in the RBS of the HA surface glycoprotein of A/Indonesia/5/2005 change the binding preference from the avian α -2,3-linked SA receptors to the human α -2,6-linked SA receptors (35). A/Indonesia/5/2005 virus with amino acid substitutions N182K, Q222L/G224S, or N182K/Q222L/G224S (numbers refer to amino acid positions in the mature H5 HA protein; N, Asn; Q, Gln; L, Leu; G, Gly; S, Ser) in HA display attachment patterns similar to those of human viruses to cells of the respiratory tract of ferrets and humans (35). Of these changes, we know that together, Q222L and G224S switch the receptor binding specificity of H2 and H3 subtype influenza viruses, as this switch contributed to the emergence of the 1957 and 1968 pandemics (36). N182K has been found in a human case of A/H5N1 virus infection (37).

Our experimental rationale to obtain transmissible A/H5N1 viruses was to select a mu-

tant A/H5N1 virus with receptor specificity for α -2,6-linked SA shed at high titers from the URT of ferrets. Therefore, we used the QuickChange multisite-directed mutagenesis kit (Agilent Technologies, Amstelveen, the Netherlands) to introduce amino acid substitutions N182K, Q222L/G224S, or N182K/Q222L/G224S in the HA of wild-type (WT) A/Indonesia/5/2005, resulting in A/H5N1_{HA N182K}, A/H5N1_{HA Q222L,G224S}, and A/H5N1_{HA N182K,Q222L,G224S}. Experimental details for experiments 1 to 9 are provided in the supplementary materials (25). For experiment 1, we inoculated these mutant viruses and the A/H5N1_{wildtype} virus intranasally into groups of six ferrets for each virus (fig. S3). Throat and nasal swabs were collected daily, and virus titers were determined by end-point dilution in Madin Darby canine kidney (MDCK) cells to quantify virus shedding from the ferret URT. Three animals were euthanized after day 3 to enable tissue sample collection. All remaining animals were euthanized by day 7 when the same tissue samples were taken. Virus titers were determined in the nasal turbinates, trachea, and lungs collected post-mortem from the euthanized ferrets. Throughout the duration of experiment 1, ferrets inoculated intranasally with A/H5N1_{wildtype} virus produced high titers in nose and throat swabs—up to 10 times more than A/H5N1_{HA Q222L,G224S}, which yielded the highest virus titers of all three mutants during the 7-day period (Fig. 1). However, no significant difference was observed between the virus shedding of ferrets inoculated with A/H5N1_{HA Q222L,G224S} or A/H5N1_{HA N182K} during the first 3 days when six animals per group were present. Thus, of the viruses with specificity for α -2,6-linked SA, A/H5N1_{HA Q222L,G224S} yielded the highest virus titers in the ferret URT (Fig. 1).

As described above, amino acid substitution E627K in PB2 is one of the most consistent host-range determinants of influenza viruses (29–31). For experiment 2 (fig. S4), we introduced E627K into the PB2 gene of A/Indonesia/5/2005 by site-directed mutagenesis and produced the recombinant virus A/H5N1_{HA Q222L,G224S PB2 E627K}. The introduction of E627K in PB2 did not significantly affect virus shedding in ferrets, because virus titers in the URT were similar to those seen in A/H5N1_{HA Q222L,G224S}-inoculated animals [up to 1×10^4 50% tissue culture infectious doses (TCID₅₀)] (Mann-Whitney *U* rank-sum test, $P = 0.476$) (Fig. 1 and fig. S5). When four naïve ferrets were housed in cages adjacent to those with four inoculated animals to test for airborne transmission as described previously (27), A/H5N1_{HA Q222L,G224S PB2 E627K} was not transmitted (fig. S5).

Because the mutant virus harboring the E627K mutation in PB2 and Q222L and G224S in HA did not transmit in experiment 2, we designed an experiment to force the virus to adapt to replication in the mammalian respiratory tract and to select virus variants by repeated

passage (10 passages in total) of the constructed A/H5N1_{HA Q222L,G224S PB2 E627K} virus and A/H5N1_{wildtype} virus in the ferret URT (Fig. 2 and fig. S6). In experiment 3, one ferret was inoculated intranasally with A/H5N1_{wildtype} and one ferret with A/H5N1_{HA Q222L,G224S PB2 E627K}. Throat and nose swabs were collected daily from live animals until 4 days postinoculation (dpi), at which time the animals were euthanized to collect samples from nasal turbinates and lungs. The nasal turbinates were homogenized in 3 ml of virus-transport medium, tissue debris was pelleted by centrifugation, and 0.5 ml of the supernatant was subsequently used to inoculate the next ferret intranasally (passage 2). This procedure was repeated until passage 6.

From passage 6 onward, in addition to the samples described above, a nasal wash was also collected at 3 dpi. To this end, 1 ml of phosphate-buffered saline (PBS) was delivered dropwise to the nostrils of the ferrets to induce sneezing. Approximately 200 μ l of the “sneeze” was collected in a Petri dish, and PBS was added to a final volume of 2 ml. The nasal-wash samples were used for intranasal inoculation of the ferrets for the subsequent passages 7 through 10. We changed the source of inoculum during the course of the experiment, because passaging nasal washes may facilitate the selection of viruses that were secreted from the URT. Because influenza viruses mutate rapidly, we anticipated that 10 passages would be sufficient for the virus to adapt to efficient replication in mammals.

Virus titers in the nasal turbinates of ferrets inoculated with A/H5N1_{wildtype} ranged from $\sim 1 \times 10^5$ to 1×10^7 TCID₅₀/gram tissue throughout 10 serial passages (Fig. 3A and fig. S7). In ferrets inoculated with A/H5N1_{HA Q222L,G224S PB2 E627K} virus, a moderate increase in virus titers in the nasal turbinates was observed as the passage number increased. These titers ranged from 1×10^4 TCID₅₀/gram tissue at the start of the experiment to 3.2×10^5 to 1×10^6 TCID₅₀/gram tissue in the final passages (Fig. 3A and fig. S7). Notably, virus titers in the nose swabs of animals inoculated with A/H5N1_{HA Q222L,G224S PB2 E627K} also increased during the successive passages, with peak virus shedding of 1×10^5 TCID₅₀ at 2 dpi after 10 passages (Fig. 3B). These data indicate that A/H5N1_{HA Q222L,G224S PB2 E627K} was developing greater capacity to replicate in the ferret URT after repeated passage, with evidence for such adaptation becoming apparent by passage number 4. In contrast, virus titers in the nose swabs of the ferrets collected at 1 to 4 dpi throughout 10 serial passages with A/H5N1_{wildtype} revealed no changes in patterns of virus shedding.

Passaging of influenza viruses in ferrets should result in the natural selection of heterogeneous mixtures of viruses in each animal with a variety of mutations: so-called viral quasi-species (38). The genetic composition of the viral quasi-species present in the nasal washes

of ferrets after 10 passages of A/H5N1_{wildtype} and A/H5N1_{HA Q222L,G224S PB2 E627K} was determined by sequence analysis using the 454/Roche GS-FLX sequencing platform (Roche, Woerden, the Netherlands) (tables S1 and S2). The mutations introduced in A/H5N1_{HA Q222L,G224S PB2 E627K} by reverse genetics remained present in the virus population after 10 consecutive passages at a frequency $>99.5\%$ (Fig. 4 and table S1). Numerous additional nucleotide substitutions were detected in all viral gene segments of A/H5N1_{wildtype} and A/H5N1_{HA Q222L,G224S PB2 E627K} after passaging, except in segment 7 (tables S1 and S2). Of the 30 nucleotide substitutions selected during serial passage, 53% resulted in amino acid substitutions. The only amino acid substitution detected upon repeated passage of both A/H5N1_{wildtype} and A/H5N1_{HA Q222L,G224S PB2 E627K} was T156A (T, Thr; A, Ala) in HA. This substitution removes a potential N-linked glycosylation site (Asn-X-Thr/Ser; X, any amino acid) in HA and was detected in 99.6% of the A/H5N1_{wildtype} sequences after 10 passages. T156A was detected in 89% of the A/H5N1_{HA Q222L,G224S PB2 E627K} sequences after 10 passages, and the other 11% of sequences possessed the substitution N154K, which removes the same potential N-linked glycosylation site in HA.

In experiment 4 (see supplementary materials), we investigated whether airborne-transmissible viruses were present in the heterogeneous virus population generated during virus passaging in ferrets (fig. S4). Nasal-wash samples, collected at 3 dpi from ferrets at passage 10, were used in transmission experiments to test whether airborne-transmissible virus was present in the virus quasi-species. For this purpose, nasal-wash samples were diluted 1:2 in PBS and subsequently used to inoculate six naïve ferrets intranasally: two for passage 10 A/H5N1_{wildtype} and four for passage 10 A/H5N1_{HA Q222L,G224S PB2 E627K} virus.

The following day, a naïve recipient ferret was placed in a cage adjacent to each inoculated donor ferret. These cages are designed to prevent direct contact between animals but allow airflow from a donor ferret to a neighboring recipient ferret (fig. S1) (27). Although mutations had accumulated in the viral genome after passaging of A/H5N1_{wildtype} in ferrets, we did not detect replicating virus upon inoculation of MDCK cells with swabs collected from naïve recipient ferrets after they were paired with donor ferrets inoculated with passage 10 A/H5N1_{wildtype} virus (Fig. 5, A and B). In contrast, we did detect virus in recipient ferrets paired with those inoculated with passage 10 A/H5N1_{HA Q222L,G224S PB2 E627K} virus. Three (F1 to F3) out of four (F1 to F4) naïve recipient ferrets became infected as confirmed by the presence of replicating virus in the collected nasal and throat swabs (Fig. 5, C and D). A throat-swab sample obtained from recipient ferret F2, which contained the highest virus titer among the ferrets in the first transmis-

sion experiment, was subsequently used for intranasal inoculation of two additional donor ferrets. Both of these animals, when placed in the transmission cage setup (fig. S1), again transmitted the virus to the recipient ferrets (F5 and F6) (Fig. 6, A and B). A virus isolate was obtained after inoculation of MDCK cells with a nose swab collected from ferret F5 at 7 dpi. The virus from F5 was inoculated intranasally into two more donor ferrets. One day later, these animals were paired with two recipient ferrets (F7 and F8) in transmission cages, one of which (F7) subsequently became infected (Fig. 6, C and D).

We used conventional Sanger sequencing to determine the consensus genome sequences of viruses recovered from the six ferrets (F1 to F3 and F5 to F7) that acquired virus via airborne transmission (Fig. 4 and table S3). All six samples still harbored substitutions Q222L, G224S, and E627K that had been introduced by reverse genetics. Surprisingly, only two additional amino acid substitutions, both in HA, were consistently detected in all six airborne-transmissible viruses: (i) H103Y (H, His; Y, Tyr), which forms part of the HA trimer interface, and (ii) T156A, which is proximal but not immediately adjacent to the RBS (fig. S8). Although we observed several other mutations, their occurrence was not consistent among the airborne viruses, indicating that of the heterogeneous virus populations generated by passaging in ferrets, viruses with different genotypes were transmissible. In addition, a single transmission experiment is not sufficient to select for clonal airborne-transmissible viruses because, for example, the consensus sequence of virus isolated from F6 differed from the sequence of parental virus isolated from F2.

Together, these results suggest that as few as five amino acid substitutions (four in HA and one in PB2) may be sufficient to confer airborne transmission of HPAI A/H5N1 virus between mammals. The airborne-transmissible virus isolate with the least number of amino acid substitutions, compared with the A/H5N1_{wildtype}, was recovered from ferret F5. This virus isolate had a total of nine amino acid substitutions; in addition to the three mutations that we introduced (Q222L and G224S in HA and E627K in PB2), this virus harbored H103Y and T156A in HA, H99Y and I368V (I, Ile; V, Val) in PB1, and R99K (R, Arg) and S345N in NP (table S3). Reverse genetics will be needed to identify which of the five to nine amino acid substitutions in this virus are essential to confer airborne transmission.

During the course of the transmission experiments with the airborne-transmissible viruses, ferrets displayed lethargy, loss of appetite, and ruffled fur after intranasal inoculation. One of eight inoculated animals died upon intranasal inoculation (Table 1). In previously published experiments, ferrets inoculated intranasally with WTA/Indonesia/5/2005 virus at a dose of 1×10^6 TCID₅₀ showed neurological disease and/or

death (39, 40). It should be noted that inoculation of immunologically naïve ferrets with a dose of 1×10^6 TCID₅₀ of A/H5N1 virus and the subsequent course of disease is not representative of the natural situation in humans. Importantly, although the six ferrets that became infected via respiratory droplets or aerosol also displayed lethargy, loss of appetite, and ruffled fur, none of these animals died within the course of the experiment. Moreover, previous infections of humans with seasonal influenza viruses are likely to induce heterosubtypic immunity that would offer some protection against the development of severe disease (41, 42). It has been shown that mice and ferrets previously infected with an A/H3N2 virus are clinically protected against intranasal challenge infection with an A/H5N1 virus (43, 44).

After intratracheal inoculation (experiment 5; fig. S9), six ferrets inoculated with 1×10^6 TCID₅₀ of airborne-transmissible virus F5 in a 3-ml volume of PBS died or were moribund at day 3. Intratracheal inoculations at such high doses do not represent the natural route of infection and are generally used only to test the ability of viruses to cause pneumonia (45), as is done for vaccination-challenge studies. At necropsy, the six ferrets revealed macroscopic lesions affecting 80 to 100% of the lung parenchyma with average virus titers of 7.9×10^6 TCID₅₀/gram lung (fig. S10). These data are similar to those described previously for A/H5N1_{wildtype} in ferrets (Table 1). Thus, although the airborne-transmissible virus is lethal to ferrets upon intratracheal inoculation at high doses, the virus was not lethal after airborne transmission.

To test the effect of the mutations in HA in the airborne-transmissible virus on its sensitivity to antiviral drugs, we used virus isolated from F5 (experiment 6). This airborne-transmissible virus with nine amino acid substitutions displayed a sensitivity to the antiviral drug oseltamivir similar to that of A/H5N1_{wildtype} (table S4).

In experiment 7, we evaluated the recognition of the airborne-transmissible virus by antisera raised against potential A/H5N1 vaccine strains. Because only HA recognition by antibodies is evaluated in this assay, chimeric viruses were generated based on six gene segments of the mouse-adapted A/Puerto Rico/8/34 (PR8) virus with the HA and PB2 genes of the transmissible virus harboring amino acid substitutions H103Y, T156A, Q222L, and G224S in HA and E627K in PB2. We replaced the MBS of the HA by a monobasic cleavage site, allowing us to do these experiments under BSL2 conditions. The chimeric PR8/H5 virus reacted well with ferret antisera raised against A/Indonesia/5/2005 and several other pre-pandemic vaccine strains (table S5). In fact, the presence of the four HA mutations increased the reactivity with H5 antisera by twofold or more.

We subsequently used the same PR8/H5 chimeric virus in experiment 8 to evaluate the presence of existing immunity against the airborne-transmissible virus in sera obtained from human

volunteers more than 70 years of age. The introduction of receptor-binding site mutations Q222L/G224S and the mutations H103Y and T156A in HA, acquired during ferret passage, did not result in increased cross-reactivity with human antisera (table S6), indicating that humans do not have antibodies against the HA of the airborne-transmissible A/H5N1 virus that was selected in our experiments.

Substitutions Q222L and G224S have previously been shown to be sufficient to switch receptor-binding specificity of avian influenza strains (i.e., α -2,3-linked SA) to that of human strains (i.e., α -2,6-linked SA) (20, 35, 46, 47). Amino acid position 103 is distal from the RBS, forms part of the trimer interface, and is unlikely to affect receptor specificity (fig. S8). T156 is part of a N-glycosylation sequon, and T156A (as well as N154K) would delete this potential glycosylation site (fig. S8); amino acid T156 is proximal but not immediately adjacent to the RBS. Loss of N-glycosylation sites at the tip of HA has been shown to affect receptor binding of A/H1 (48, 49) and the virulence of A/H5 virus (50). We evaluated the impact of the HA mutations that emerged during passaging in ferrets in a modified turkey red blood cell (TRBC) assay (Table 2). In this assay, the binding of influenza viruses, with a mutated HA, to normal TRBCs (expressing both α -2,3-linked SA and α -2,6-linked SA) and modified TRBCs with either α -2,3-linked SA or α -2,6-linked SA on the cell surface was evaluated and compared to two reference viruses with known receptor binding preference: avian A/H5N1 and human A/H3N2 viruses. As expected and shown before, introduction of the Q222L and G224S mutations in the HA of A/H5N1 changed the receptor binding preference from α -2,3-linked SA to α -2,6-linked SA (35). Furthermore, in our hands, the introduction of substitutions H103Y and T156A not only enhanced binding of A/H5N1_{HA Q222L,G224S} PB2 E627K to α -2,6-linked SA, as expected from glycan array studies (51), but also increased the affinity for α -2,3-linked SA. When these two mutations were introduced in the A/H5N1_{wildtype} HA, the affinity for α -2,3-linked SA also increased.

Substitutions Q222L and G224S have previously emerged in avian A/H2 and A/H3 viruses in nature (36, 52), and mutations associated with similar changes in receptor binding specificity have been detected repeatedly in A/H5 viruses—for instance, substitution N182K has been reported nine times (37, 51), which is why we initially selected it for our investigations. The other three substitutions we found consistently in airborne-transmissible viruses have all previously been detected in HPAI A/H5N1 viruses circulating in the field (53). Only a minor fraction of the A/H5N1 viruses that have circulated in outbreaks have been sequenced (estimated to be <0.001%) (53, 54). Yet the individual substitutions we obtained, as well as combinations of T156A and

H103Y or T156A and E627K, have already been reported in public sequence databases (53); thus, we conclude that these mutations do not appear to have a detrimental effect on virus fitness. Substitution H103Y has only been found once, in combination with T156A in a duck in China (53). Substitution E627K in PB2 has been found in ~27% of avian A/H5N1 virus sequences and in ~29% of human A/H5N1 viruses (53). Substitution T156A in HA has been reported in >50% of the viruses sequenced and was detected in 100% of the viruses from human cases in Egypt (53).

Investigations of viral quasi-species during a massive avian influenza A/H7N7 virus outbreak in the Netherlands indicated that viruses with human adaptation markers, including HA mutations that alter receptor specificity and mutations in polymerase proteins that increase polymerase activity like E627K in PB2, emerged rapidly in poultry (55–57). Given the large numbers of HPAI A/H5N1 virus-infected hosts globally, the high viral mutation rate, and the apparent lack of detrimental effects on fitness of the mutations that confer airborne transmission, it may simply be a matter of chance and time before a human-to-human transmissible A/H5N1 virus emerges.

The specific mutations we identified in these experiments that are associated with airborne transmission represent biological traits that may be determined by a set of different amino acid substitutions. For example, amino acid substitutions D701N (D, Asp) or S590G/R591Q in PB2 yield a similar phenotype to E627K (29). N182K and other substitutions in the RBS of HA may yield a similar phenotype to Q222L/G224S (35). Such mutations should be considered for A/H5N1 surveillance studies in outbreak areas. Imai *et al.* recently identified different RBS changes (N220K, Q222L) along with N154D (affecting the same N-glycosylation sequon as T156A) and T314I in HA as determinants of airborne transmission of an A/H5 virus (58). This airborne virus contained seven genes of the 2009 pandemic A/H1N1 virus (which has S590G/R591Q in PB2 rather than E627K), with the HA of A/H5N1 virus A/Vietnam/1203/2004 (58). These data indicate that different lineages of A/H5N1 virus and different amino acid substitutions that affect particular biological traits (receptor binding, glycosylation, replication) can yield airborne-transmissible A/H5N1 viruses.

Although our experiments showed that A/H5N1 virus can acquire a capacity for airborne transmission, the efficiency of this mode remains unclear. Previous data have indicated that the 2009 pandemic A/H1N1 virus transmits efficiently among ferrets and that naïve animals shed high amounts of virus as early as 1 or 2 days after exposure (27). When we compare the A/H5N1 transmission data with that of reference (27), keeping in mind that our experimental design for studying transmission is not quantitative, the data shown in Figs. 5 and 6 suggest that A/H5N1 airborne transmission was less robust, with less

and delayed virus shedding compared with pandemic A/H1N1 virus.

Airborne transmission could be tested in a second mammalian model system such as guinea pigs (59), but this would still not provide conclusive evidence that transmission among humans would occur. The mutations we identified need to be tested for their effect on transmission in other A/H5N1 virus lineages (60), and experiments are needed to quantify how they affect viral fitness and virulence in birds and mammals. For pandemic preparedness, antiviral drugs and vaccine candidates against airborne-transmissible virus should be evaluated in depth. Mechanistic studies on the phenotypic traits associated with each of the identified amino acid substitutions should provide insights into the key determinants of airborne virus transmission. Our findings indicate that HPAI A/H5N1 viruses have the potential to evolve directly to transmit by aerosol or respiratory droplets between mammals, without reassortment in any intermediate host, and thus pose a risk of becoming pandemic in humans. Identification of the minimal requirements for virus transmission between mammals may have prognostic and diagnostic value for improving pandemic preparedness (34).

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Acknowledgments: We thank D. de Meulder, G. van Amerongen, and D. Akkermans for technical assistance. M. Peiris, Univ. of Hong Kong, provided A/Indonesia/5/2005 with permission from I. Kandon of the Indonesian government. This work was financed through NIAID-NIH contract HHSN266200700010C. D.J.S. and D.F.B. were supported in part by NIH Director's Pioneer Award DP1-OD000490-01. We acknowledge a Nederlandse Organisatie voor Wetenschappelijk Onderzoek VICI grant, European Union FP7 program EMPIRE (223498), and Human Frontier Science Program grant P0050/2008. D.F.B. and D.J.S. acknowledge the use of the CamGrid distributed computing resource. Sequence data generated from this study were deposited in GenBank with accession numbers CY116643 to CY116698. Special arrangements are in place with the NIH and the contractor at Mount Sinai School of Medicine, New York, for sharing the viruses (and plasmids) in the present paper; please contact R.A.M.F. A.D.M. E.O. and G.F.R. are CSO and part-time employee of ViroClinics Biosciences BV. A.D.M.E.O. has advisory affiliations on behalf of ViroClinics Biosciences BV with GlaxoSmithKline, Novartis, and Roche. A.D.M.E.O. and R.A.M.F. are holders of certificates of shares in ViroClinics Biosciences B.V. To avoid any possible conflict of interests, Erasmus MC policy dictates that the shares as such are held by the Stichting Administratiekantoor Erasmus Personeelsparticipaties. The board of this foundation is appointed by the Board of Governors of the Erasmus MC and exercises all voting rights with regard to these shares.

Supplementary Materials

www.sciencemag.org/cgi/content/full/336/6088/1534/DC1
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30 August 2011; accepted 31 May 2012
10.1126/science.1213362

REPORT

The Potential for Respiratory Droplet–Transmissible A/H5N1 Influenza Virus to Evolve in a Mammalian Host

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Avian A/H5N1 influenza viruses pose a pandemic threat. As few as five amino acid substitutions, or four with reassortment, might be sufficient for mammal-to-mammal transmission through respiratory droplets. From surveillance data, we found that two of these substitutions are common in A/H5N1 viruses, and thus, some viruses might require only three additional substitutions to become transmissible via respiratory droplets between mammals. We used a mathematical model of within-host virus evolution to study factors that could increase and decrease the probability of the remaining substitutions evolving after the virus has infected a mammalian host. These factors, combined with the presence of some of these substitutions in circulating strains, make a virus evolving in nature a potentially serious threat. These results highlight critical areas in which more data are needed for assessing, and potentially averting, this threat.

Recent studies have shown that the A/Indonesia/5/2005 avian A/H5N1 influenza virus may require as few as five

amino acid substitutions (1), and the A/Vietnam/1203/2004 A/H5N1 influenza virus requires four substitutions and reassortment (2), to become

transmissible between ferrets via respiratory droplets. Here, we assess the likelihood that these substitutions could arise in nature. We first analyzed A/H5N1 sequence surveillance data to identify whether any of these substitutions are already circulating. We then explored the probability of the virus evolving the remaining substitutions after a spillover event of an avian virus into a single mammalian host and in a short chain of transmission between mammalian hosts.

The minimal set of substitutions identified by (1) (the Herfst *et al.* set) contains two receptor-binding amino acid substitutions, Q222L and G224S (H5 numbering used throughout) in the hemagglutinin (HA), known to change the virus from the more avian-like alpha-2-3-linked sialic acid specificity to the more humanlike alpha-2-6-linked sialic acid (3, 4). The remaining three substitutions in the set are T156A in HA, which disrupts the N-linked glycosylation sequon spanning positions 154 to 156; H103Y in the HA trimer-interface; and E627K in the PB2, which is a common mammalian polymerase adaptation (5). (Numbers refer to amino acid positions in the mature H5 proteins; for example, Q222L indicates that glutamine at position 222 was replaced by leucine. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.)

The four amino acid substitutions in HA identified by (2) (the Imai *et al.* set) also contain two receptor-binding amino acid substitutions, N220K and Q222L, one of which is in common with the Herfst *et al.* set and which together are known to change the sialic acid linkage preference to the more human-like alpha-2-6 linkage (2). The remaining two substitutions are N154D, which dis-

rupts the same N-linked glycosylation sequon as the T156A substitution in the Herfst *et al.* set, and T315I in the stalk region.

Of the three receptor-binding substitutions in the two sets, only N220K in the Imai *et al.* set has been detected by means of surveillance in consensus sequencing of the HA of A/H5N1 viruses, and only in 2 of 3392 sequences [both avian viruses, one from 2007 in Vietnam, one from Egypt in 2010 (Fig. 1, B and F, black arrows)]. The T315I stalk substitution and H103Y trimer interface substitution have each been detected once in two viruses from China in 2002 (Fig. 1, A and B, orange arrows). T315I has been detected in two pre-1997 H5N1 viruses, four H5N2 viruses, two H5N3 viruses, and two H5N9 viruses. H103Y has been detected in five H5N2 viruses and one H5N3 virus. The remaining substitutions, N154D and T156A in the HA glycosylation sequon and E627K in PB2, however, are common and occur in 942 of 3392, 1803 of 3392, and 432 of 1612 sequences, respectively. A summary of the substitutions detected in surveillance is shown in fig. S1 and table S1. For viruses in which both HA and PB2 have been sequenced, 338 of 1533 have lost the 154-to-156 glycosylation sequon and have E627K in PB2. These viruses have been collected in at least 28 countries in Europe, the Middle East, Africa, and Asia.

The HA glycosylation sequon substitutions, N154D and T156A, have drifted in and out of the avian virus population over time, suggesting that they may be under little selective pressure in birds. The other substitutions—which are rare in birds, particularly those that change the sialic acid linkage preference—are likely to be negatively selected in birds.

Phylogenetic trees of the A/H5N1 HA are shown in Fig. 1, color-coded by the number of nucleotide mutations required to obtain the five Herfst *et al.* set (column 1) and four Imai *et al.* set (column 2) of substitutions in HA. Obtaining these mutations does not necessarily mean the virus will be transmissible through respiratory droplets between ferrets because the genetic background of each strain is different from the strain used by Herfst *et al.* (Fig. 1A, blue circle) and the strain used by Imai *et al.* (Fig. 1J, red circle). Other than for clade 2.3.2.1, the variation in color in Fig. 1, columns 1 and 2, is due to the presence (mostly in East and Southeast Asia) or absence (mostly outside of East and Southeast Asia) of the glycosylation sequon at positions 154 to 156.

The sequenced viruses that are closest to the Herfst *et al.* set are in clade 2.3.2.1 (Fig. 1A and fig. S1A). These HAs have acquired a silent nucleotide mutation that makes the amino acid substitution G224S require only one nucleotide mutation instead of the two mutations for other strains. It is the requirement of these two nucleotide mutations that makes viruses usually far-

ther from the Herfst *et al.* set than the Imai *et al.* set. The viruses in clade 2.3.2.1 have been sampled in Nepal, Mongolia, Japan, and Korea from 2009 to 2011. Seventeen out of 94 of these viruses have been sequenced in PB2 (Fig. 1D), and none have the E627K substitution. Thus, the closest known viruses to the Herfst *et al.* set by consensus sequencing are four nucleotide substitutions away.

The majority of H5 viruses in clade 2.2 (and its subclades) are three nucleotide mutations from the Imai *et al.* set in HA (Fig. 1, F and J). These viruses have been sampled in Europe from 2005 to 2007, in the Middle East (including Egypt) from 2005 to 2011, and in Africa from 2005 to 2007. Viruses sampled in 2010 and 2011 are indicated by the red portion of the vertical line delimiting the clade (Fig. 1 and by the time series in fig. S1, F and J). If it is the loss of glycosylation that is important, rather than any other effect of N154D, then as shown in Fig. 1, column 3, almost all the non-Asian viruses have lost the glycosylation sequon, and thus all these viruses would potentially be functionally three nucleotides from the Imai *et al.* set in HA.

The viruses indicated by the black arrows in Fig. 1, B and F (one from Vietnam in 2007 and one from Egypt in 2010), have the N220K receptor-binding substitution and have lost the glycosylation sequon at positions 154 to 156. Thus, these two viruses are two nucleotide substitutions from the Imai *et al.* set in HA, and are the viruses closest to having the full Imai *et al.* set in HA detected to date by means of consensus sequencing.

Surveillance has detected humans with A/H5N1 viruses four nucleotide mutations from the full Herfst *et al.* set and three from the Imai *et al.* set in HA. Viruses isolated from human A/H5N1 infections (Fig. 1, bottom row) are generally the same number of mutations in HA away from the Herfst *et al.* and Imai *et al.* sets, by means of consensus sequencing, as their most closely related avian viruses. The within-host evolution modeling below indicates that any host adaptation substitutions would only reach a small proportion of the total virus population in the first spillover host and, although potentially critical in the host-adaptation process, would not be detected with consensus sequencing. Thus, the absence of evidence of host-adaptation through consensus sequencing is not evidence for the absence of potentially critical adaptation to the mammalian host. See (6) for details of human strains and their most genetically similar avian A/H5N1 viruses.

To explore the probability of accumulating the remaining nucleotide mutations after the avian virus has been transmitted to a human (or other mammalian host), we constructed a mathematical model (7–10) of the within-host evolutionary dynamics of the virus. In the model, errors made by the virus polymerase are the source of mutation

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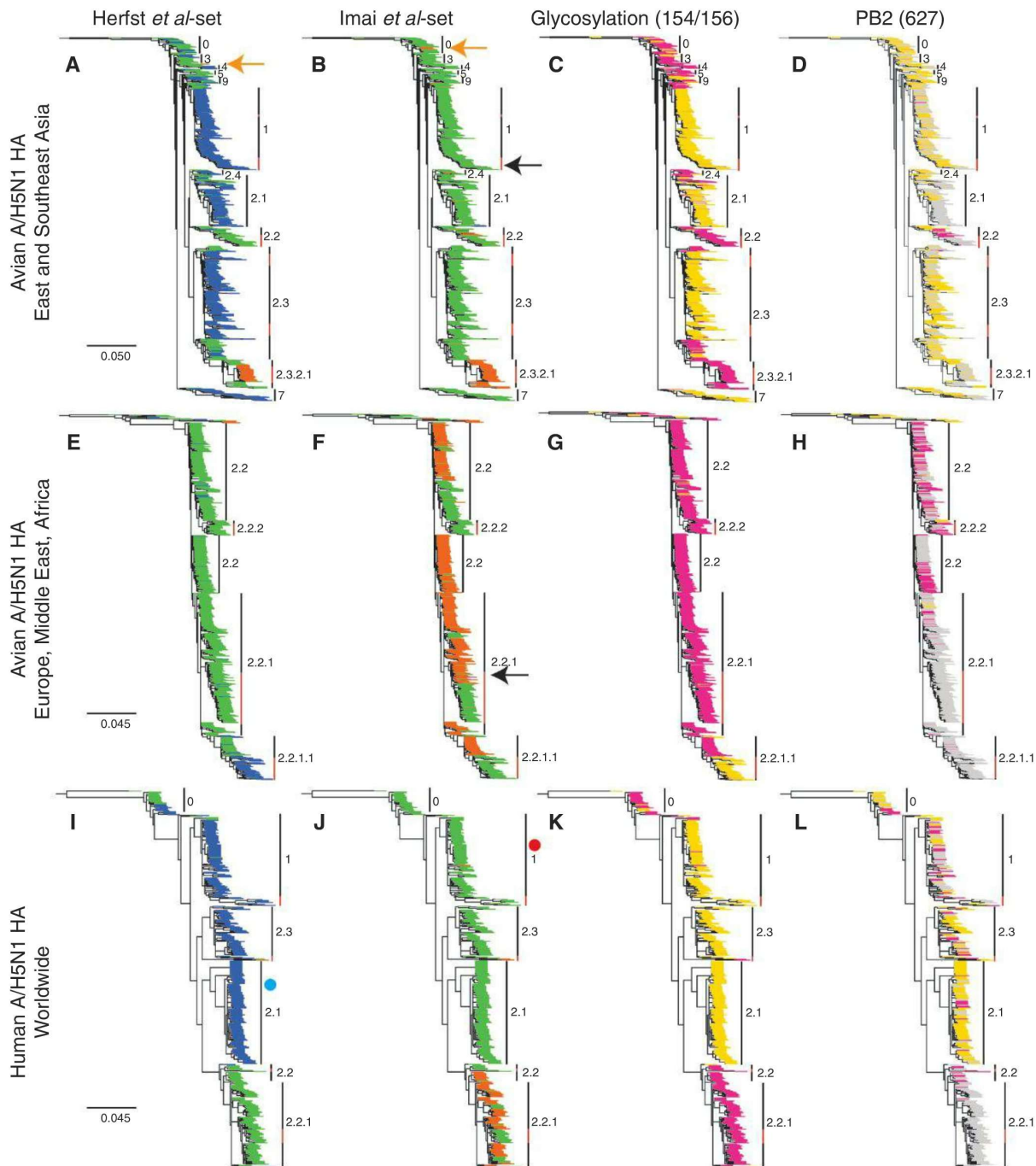


Fig. 1. (A to L) Phylogenetic trees of the A/H5N1 HA1 nucleotide sequences. The sequences are split into three trees: 2022 avian H5 sequences from East and Southeast (E and SE) Asia (top row); 1097 avian H5 sequences from Europe, the Middle East, and Africa (middle row); and 385 human H5 sequences (bottom row). Each sequence is color coded by the minimum number of nucleotide mutations required to obtain the four amino acid substitutions in HA in the Herfst *et al.* set (column 1), to obtain the four amino acid substitutions in the Imai *et al.* set (column 2), to disrupt the N-linked glycosylation sequon spanning positions 154 to 156 in HA (column 3), and to obtain E627K in the PB2 segment of the corresponding virus in these HA trees (column 4). In columns 1 and 2, blue indicates five nucleotide changes, green indicates four, and orange indicates three. In columns 3 and 4, yellow viruses require one mutation, and pink require zero mutations. Gray indicates PB2

not sequenced. Clades as defined by (35) are marked to the right of the branches; the red portion of the vertical clade-identification lines indicates strains sampled in 2010 or 2011. The viruses indicated by black arrows are two nucleotides from the Imai *et al.* set. The virus indicated in (A) by the orange arrow has the H103Y substitution, and the virus indicated in (B) by the orange arrow has the T315I substitution. The blue circle indicates A/Indonesia/5/2005, and the red circle indicates A/Vietnam/1203/2004, the starting viruses used by (1) and (2), respectively. The initial trees were constructed with PhyML version 3.0 (36), with A/Chicken/Scotland/1959 as the root, using GTR+I+ Γ 4 [determined by ModelTest (37)] as the evolutionary model. GARLI version 0.96 (38) was run on the best tree from PhyML for 1 million generations to optimize tree topology and branch lengths. "Zoom-able" versions of these trees are shown in fig. S1 to show detail.

(10^{-5} mutations per site, per genome replication), the initial virus population expands exponentially [each infected cell produces 10^4 virions (11, 12), and 10^{10} cells can be infected (13)] until it reaches 10^{14} virions, after which the virus population size stays roughly constant, and selection is modeled by use of differences in expected numbers of progeny (fig. S2 and table S2) (6). The results

of the model are largely insensitive to the number of cells that can be infected, maximum virus population size, and whether the virus population remains roughly constant or declines (figs. S3 to S5). Typical infections were simulated out to 5 days corresponding to the approximate time of peak viral load, and long-duration infections to 14 days (14).

It is not possible to calculate the level of risk precisely because of uncertainties in some aspects of the biology. We used the model to compare the relative effects of factors that could increase or decrease the probability of accumulating mutations and to identify areas for further investigation that are critical for more accurate risk assessment. We compare and contrast the effects of factors that can increase the probability of accumulating mutations and thus evolving a respiratory droplet-transmissible A/H5N1 influenza virus in a mammalian host, and factors that could decrease the probability of evolving a such a virus. The factors we considered that can increase the probability are random mutation, positive selection, long infection, alternate functionally equivalent substitutions, and transmission of partially adapted viruses as a proportion of the within-host diversity both in the avian-mammal and the mammal-mammal transmission events (10, 14–18). The factors we considered that can decrease the probability are an effective immune response, deleterious substitutions, and order-dependence in the acquisition of substitutions. We considered these factors for starting viruses differing in the number of mutations that separates them from a respiratory droplet-transmissible A/H5N1 virus—viruses that require five, four, three, two, or one mutations at specific positions in the virus HA, reflecting that zero, one, two, three, or four of the mutations are already present in the avian population and thus are present at the start of the infection in mammals. We treat each amino acid substitution as if it can be acquired by a single-nucleotide mutation, as is the case for

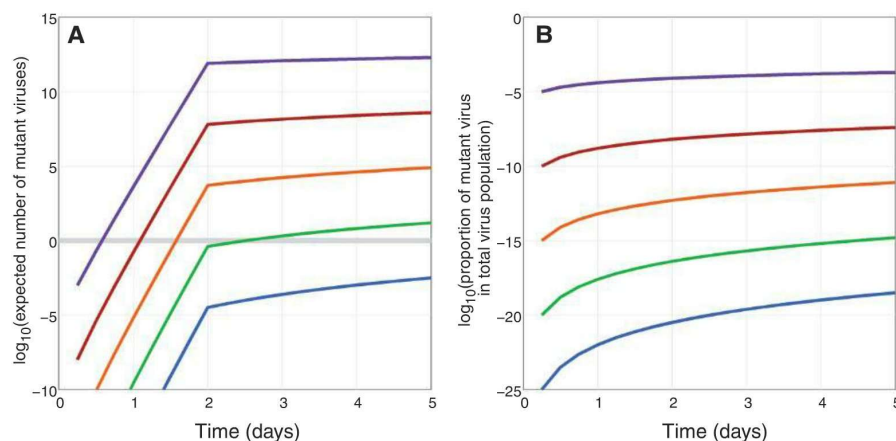


Fig. 2. Expected absolute numbers and proportions of respiratory droplet-transmissible A/H5N1 virions within a host initially infected by strains that require five (blue), four (green), three (orange), two (red), or one (purple) mutation (or mutations) to become respiratory droplet-transmissible, calculated from the deterministic model. (A) The absolute number of respiratory droplet-transmissible A/H5N1 viruses in a host. The intersections with the gray line indicate the point when at least one virus in each host is expected to have the required mutations. The change in slope is due to the transition in the virus population from exponential expansion to constant size. (B) Expected proportion of respiratory droplet-transmissible A/H5N1 viruses in the total virus population over time in the random mutation case (when all mutations are fitness-neutral).

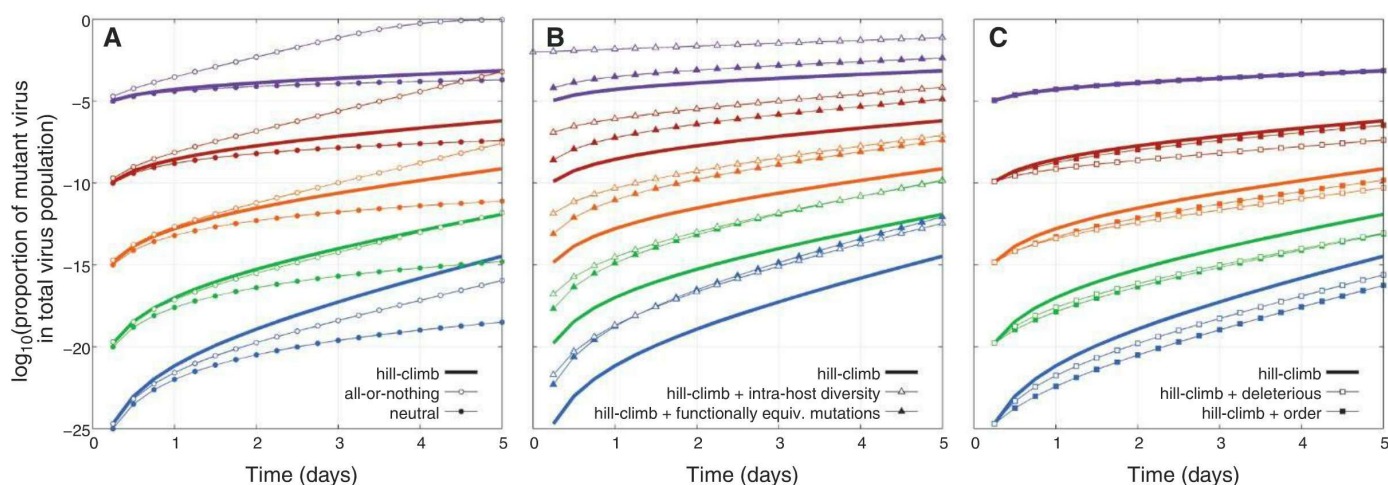


Fig. 3. Factors that increase or decrease the proportion of respiratory droplet-transmissible A/H5N1 virus based on starting viruses that require five (blue), four (green), three (orange), two (red), or one (purple) mutation (or mutations) to become respiratory droplet-transmissible. (A) The effect of hill-climb and all-or-nothing positive selection compared with random mutation alone. (B) The effect of avian-mammal transmission of partially adapted virus as a result of intra-host diversity (100 viruses start the infection, one of which has a mutation) and the effect of alternate substitutions with 10 functionally equivalent sites for a virus requiring five mutations (blue), nine

sites for a virus requiring four mutations (green), eight sites for a virus requiring three mutations (orange), seven sites for a virus requiring two mutations (red), and six sites for a virus requiring one mutation (purple), both with hill-climb selection, compared with hill-climb selection alone. (C) The effect of two of the required substitutions being individually deleterious (for these two specific substitutions, either substitution alone reduces the replicative fitness of the virus to zero) and the effect of complete order dependence of acquiring substitutions, both with hill-climb selection as compared with hill-climb selection alone.

the circulating viruses closest to acquiring the Herfst *et al.* or Imai *et al.* sets [see (6) for the general case].

First, we considered random mutation. Even without any positive selection pressure, the random process of mutations introduced by the virus polymerase in the expanding population of viruses will on average produce viruses that contain the required single, double, or triple mutations and even some quadruple mutants. These mutants will arise after a few days of an infection in a host in which the virus replicates efficiently (Fig. 2A) and would be delayed if replication is impaired (fig. S5). However, the existence of a virus within-host does not mean that it will transmit because it might exist only as a small proportion of the total virus population and thus have little chance of being excreted (Fig. 2B). The minimum proportion of mutant virus required to make transmission likely is not known, but increased proportion translates into increased probability of transmission; thus, we focused on proportion of mutant virus in the total virus population. These proportions (equivalent to the probability of a single virion to be a mutant), both here and below, cannot yet be precisely determined—they are sensitive to some biological parameters that are not yet known accurately and some that are specific to a particular virus or mutant. For such parameters, we tested a range of the current best estimates and focused on the relative, rather than the absolute, effects (6).

Second, we considered positive selection. Some of the substitutions identified by (1) and (2) have been shown to increase within-host virus fitness—specifically, the loss of glycosylation at positions 154 and 156 and E627K in PB2. However, given the absence of specific information on the within-host selective advantage or disadvantage conferred by each substitution, or combination of substitutions, we considered two cases of positive selection: one in which each individual substitution confers an additive advantage (hill-climb) and one in which only viruses

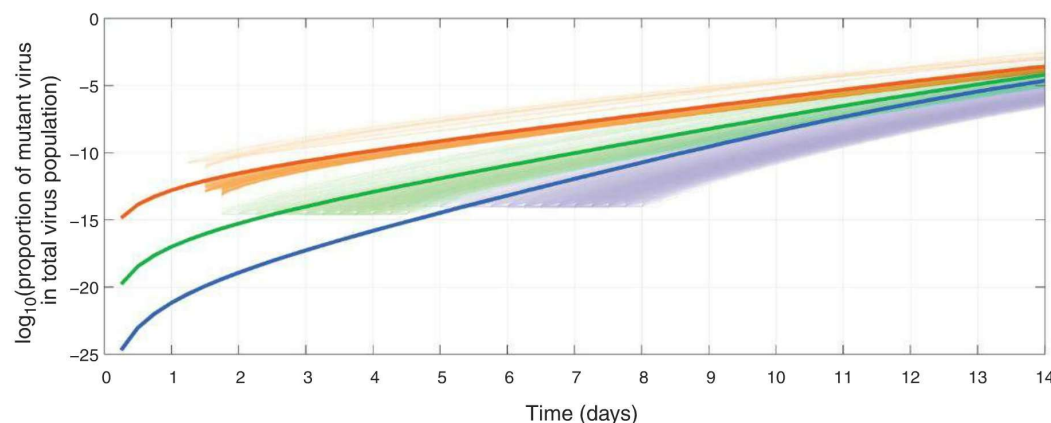
that have acquired all substitutions have an advantage (all-or-nothing). We considered a total advantage of 1.1-, 2-, or 10-fold in each genome replication step for the full set of respiratory droplet transmission-enabling substitutions (table S2 and fig. S6) (A twofold advantage at each genome replication step translates into an approximately 100-fold increase in mutant virus titer after 36 hours.) In the all-or-nothing scenario, a strong increase in proportion occurs for viruses that have acquired all mutations because of its substantial fitness advantage over the rest of the population. The rate at which all-or-nothing selection increases the proportion of respiratory droplet-transmissible A/H5N1 viruses, as compared with the neutral case, is mostly independent of the number of mutations required (Fig. 3A). In contrast, for hill-climb selection the rate of increase above the neutral case decreases when fewer mutations are required (Fig. 3A). This difference between the all-or-nothing and hill-climb is because the fitness differential from the starting virus to the respiratory droplet-transmissible A/H5N1 virus decreases as the number of needed mutations decreases (if some of the mutations are already present in the avian host) (table S2) (6). We consider this hill-climb case to be the most likely situation during the host-adaptation we modeled (in the absence of deleterious substitutions). However, we have also compared the two selection scenarios when the starting fitnesses of all-or-nothing and hill-climb are the same independent of the starting number of necessary mutations, and discuss the subtle tradeoff between the fitness advantage of, and clonal-interference among, intermediate mutants (figs. S7 and S8) (6, 19).

Third, we considered long infection. Because both random mutation and positive selection increase the expected proportion of mutated virions with every viral generation, the longer a host is infected, the greater the proportion of a particular mutant (Fig. 4) (15). Human A/H5N1 infections lasting 14 days or longer have been

reported especially in children, the elderly, and the immunocompromised (14) and have been associated with the evolution of oseltamivir resistance (20). It might be that only immunocompromised individuals can typically transmit the virus late in a long infection. The increasing proportion of mutant virus is only dependent on continued virus production and is independent of whether the virus load stays constant or declines (fig. S4) (21). The variance in the proportion of mutant virus (Fig. 4, pale regions) increases with each additional mutation required because of the increased number of combinatorial options and the greater selective advantage of mutant viruses as compared with wild-type viruses in the hill-climb scenario. The pale regions only reflect the within-model variance in results, as indicated by the different runs of the stochastic model, and not uncertainty as a result of other factors; sensitivity of the outcomes for model parameters such as the error rate and the number of virions produced by each infected cell are explored in (6) (fig. S5).

Fourth, we considered functionally equivalent substitutions. The sets of substitutions required for a respiratory droplet-transmissible A/H5N1 virus identified by (1) and (2) are unlikely to be the only combinations of substitutions capable of producing a respiratory droplet-transmissible A/H5N1 virus. If particular biological traits could be achieved by other substitutions, this would increase the expected proportions of respiratory droplet-transmissible A/H5N1 viruses. This is likely to be the case, given that there are multiple substitutions that can cause changes in receptor-binding specificity and two sites where substitutions will result in loss of glycosylation: positions 154 and 156 (table S3). If five substitutions could be from any 10 specific positions in the virus genome (or if two already existed in nature, three from any eight), then there would be 252 (or 56) combinations, and this would raise the proportion of respiratory droplet-transmissible A/H5N1 virus within a host by $\sim 10^{2.5}$ (or $\sim 10^{1.5}$) above the case of positive

Fig. 4. Proportion of respiratory droplet-transmissible A/H5N1 virus in a long infection with virus replication for 14 days in the presence of hill-climb selection. Bold lines show results from a probability-based deterministic model of virus mutation, the pale region (composed of lines) shows 10,000 stochastic model simulations for each starting virus. Starting viruses require either five (blue), four (green), or three (orange) mutations to become respiratory droplet-transmissible. For the stochastic simulations, the lines start when the first virion that has the required mutations appears.



selection alone after 5 days (Fig. 3B, figs. S9 and S10, and table S4).

Fifth, we considered the avian-to-mammal transmission of partially adapted mutants. We specifically considered the case in which one of the required mutations exists as a small proportion of the avian within-host viral population, or in the viral populations from the >20 mammalian hosts in which A/H5N1 infections have been observed (22–25), so that they would not be detected by the usual consensus sequencing techniques. If the mutant is one of the 100 virions that seed an infection (16, 17), then with positive selection the probability of acquiring the remaining mutations increases by 10^3 after 5 days of infection above the case of positive selection alone (Fig. 3B). If the proportion of mutants in the seeding population is 10^{-4} however, the increase in proportion of respiratory droplet transmissible A/H5N1 virions in the mammalian host is small (fig. S11).

Sixth, we considered mammal-to-mammal transmission of partially adapted viruses. Transmission of viruses between mammals that have some but not all of the substitutions necessary for respiratory droplet transmission potentially increases the risk of evolving a respiratory droplet-transmissible A/H5N1 virus, but this increase is modulated by the difficulty of transmitting partially adapted strains and the loss of diversity at transmission. Two primary factors strongly modulate the effect of transmission on the accumulation of mutations. First, transmission could decrease the accumulation of mutations by the loss of low-proportion mutants because only a limited portion of the virus population will be transmitted. Second, transmission could increase the accumulation of respiratory droplet transmission-enabling substitutions by concentrating a transmissible virus during excretion from or seeding into a host—for example, if the adapted virus has increased tropism for the mammalian upper respiratory tract and therefore concentrated in the nose and throat. Thus, the effect of transmission can range from negligible, if mutants are culled by the loss of diversity at transmission, to substantial, if selection favors mutants at transmission (table S5). Given that A/H5N1 virus infections have been observed in >20 mammalian species, there is a potentially large pool of nonhuman hosts in which short chains of transmission could play a role in the emergence of respiratory droplet-transmissible A/H5N1 viruses.

In contrast to these factors that could increase the rate of accumulating substitutions, we next discuss factors that could decrease this rate.

First, we considered an effective immune response. An immune response that substantially shortened an infection would decrease the probability of the accumulation of mutations; however, there are many reported cases of infections up to and beyond 5 days (14, 21). Variation in the

number of virions produced by each infected cell does not affect the deterministic calculations of the proportion of mutants. However, if this number is substantially lower for the stochastic simulations—for example, 25 (6) as compared with 10,000 (used for most of the figures)—the slower growth and lower total number of viruses could substantially delay the appearance of mutants within a host. As the number of required mutations increases, stochastic effects caused by the slower growth decrease the proportion of these mutants (fig. S5) (6).

Second, we considered deleterious intermediate substitutions. The receptor binding and trimer-interface or stalk substitutions required by (1) and (2) are, as we have seen, either rare or absent in influenza viruses isolated to date. The receptor-binding substitutions, although deleterious in birds, would be expected to be advantageous in humans. However, the details of this host-adaptation are not yet elucidated, and so we also consider the possibility that there are deleterious intermediate substitutions and explore a variety of scenarios (figs. S12 and S13). When two of the required substitutions are individually deleterious (for these two specific substitutions, either substitution alone reduces the replicative fitness of the virus to zero), this slows the rate of accumulation of mutations for the three-mutation case by less than the amount that hill-climb positive selection increases the rate above the neutral case (Fig. 3C). When three substitutions are required (all single and double substitutions reduce the replicative fitness of a mutant virus to zero), this can lower the accumulation rate $\sim 10^2$ below the neutral case (fig. S12). Deleterious (or advantageous) substitutions other than the respiratory droplet-transmissible A/H5N1 substitutions can, to a first approximation, be ignored in calculating proportions because such substitutions would on average affect all viruses equally and thus would not specifically affect the accumulation of respiratory droplet-transmissible A/H5N1 mutations (6).

Third, we considered order dependence in the acquisition of substitutions. It is not currently known whether the acquisition of some or all of the respiratory droplet transmission-enabling substitutions is dependent on the order in which viruses accumulate those substitutions. For example, the gain of 2-6-receptor binding might be required before loss of 2-3-receptor binding. If there were any order dependence, it would slow down the rate of accumulation of mutations. However, even in the most extreme scenario in which there is a single specific order in which the substitutions must be acquired, and any other order results in a virion with a replicative fitness of zero, if fewer than four mutations are required, the effect on the rate of accumulation of mutations is less than that of the deleterious scenario described above (Fig. 3C and figs. S14 and S15).

In addition to the substitutions in HA, the Imai *et al.* virus was a reassortment with an A/H1pdm09 virus. The probability of a reassortment event is difficult to determine given current knowledge. In one study (26), it has been estimated to be more likely than the likelihood of acquiring a single mutation as calculated here.

Highly pathogenic avian A/H5N1 viruses have been infecting humans for over a decade, with ~ 600 reported cases to date (and possibly many more that have not been reported), but there have yet to be known cases of efficient human-to-human transmission (27, 28). One hypothesis for the lack of sustained transmission is that it is not possible for A/H5N1 viruses to become respiratory droplet-transmissible in mammals; (1) and (2) have shown that this may not be the case in ferrets. Another hypothesis is that the number of mutations necessary for respiratory droplet-transmissibility might be so great that such a virus would be unlikely to evolve. We show here that in biologically plausible scenarios, respiratory droplet-transmissible A/H5N1 viruses can evolve during a mammalian infection. Given that respiratory droplet transmission between mammals is possible and that respiratory droplet-transmissible A/H5N1 mutants are likely to evolve in infected individuals, the primary impediment to transmission could be whether the respiratory droplet-transmissible A/H5N1 viruses comprise a sufficient proportion of the within-host viral population to actually transmit.

The minimum proportion of virus required for transmission is not known, but increased proportion likely translates into increased probability of transmission. There cannot be respiratory droplet transmission if there are no viruses in the air. Given a peak excretion rate of $\sim 10^7$ viruses per day (29, 30), a proportion of which are likely to become aerosolized (31), mutants at proportions near or above 10^{-7} might thus be among the particles excreted. Each of the factors analyzed above has a potentially substantial effect on the rate of accumulating mutations (Fig. 3), and the effects of each can be additive. With plausible combinations of these factors, a virus that requires three mutations reaches proportions at which a few respiratory droplet-transmissible A/H5N1 viruses are likely to be among the particles excreted. For a virus that requires five mutations, it may only reach such proportions with more extreme combinations of factors or if an event occurs that is not encompassed by the model (32). However, it is known that influenza viruses are capable of respiratory droplet transmission in animal models at low infectious doses (33), and that transmission routes other than in respiratory droplets could be important; thus despite the three key current unknowns about transmission (6), even low numbers of excreted respiratory droplet-transmissible A/H5N1 virus may be relevant for emergence. In addition, the probability of emer-

gence increases when more mammals are infected when this also corresponds with a rise in potential transmission events. The output of the model is a guide to understand the approximate effects of different factors and should not be interpreted as actual proportions of virus and probabilities of transmission, given the uncertainty inherent in parameter estimates and model structure, and the inherent unpredictability of rare events (34).

These results highlight four areas of investigation that are critical to more accurately assess and monitor the risk of a respiratory droplet-transmissible A/H5N1 virus emerging and to increase our understanding of virus emergence in general. Some of this work is already ongoing, planned, or suggested. The work of Herfst *et al.* (1) and Imai *et al.* (2) and the analyses here help to prioritize particular areas.

First, additional surveillance in higher-risk regions where viruses require fewer nucleotide mutations to acquire respiratory droplet transmission-enabling substitutions (Fig. 1 and fig. S1) (and in regions connected by travel, trade, and migratory flyways) is key for monitoring the emergence of a respiratory droplet-transmissible A/H5N1 virus. Surveillance of nonhuman mammalian hosts, especially any that harbor long infections or live in large groups, is important for the early identification of mammalian adaptation. Additionally, studies are needed on the accumulation of mutations within-host and in short chains of transmission in mammals (22–25), even when endemic circulation has not been observed.

Second, deep sequencing of avian and other nonhuman virus samples is necessary to accurately estimate the prevalence of the respiratory droplet transmission-enabling amino acid substitutions in nature. Deep sequencing of human samples, particularly at multiple time points from individuals with long infections, would be useful for evaluating within-host evolution, for estimating selective advantage of substitutions, and for testing the underlying dynamics and assumptions of the model (15). Respiratory droplet-transmissible A/H5N1 mutations present in a proportion higher than the polymerase error rate—exceeding approximately 10^{-5} , but far below the threshold for detection with consensus sequencing and thus not detectable with current surveillance practices—would increase the risk of a respiratory droplet-transmissible A/H5N1 evolving. Thus, sequencing deeper than that currently routinely achieved for RNA viruses (ideally detecting mutations at 0.1% frequency and lower for detailed studies) is necessary to more accurately assess the risk posed by intra-host variability (15).

Third, experiments are needed to determine which substitutions, besides the already identified receptor-binding substitutions by (1) and (2), are capable of producing respiratory droplet-transmissible A/H5N1 viruses, including the important case of functionally equivalent substitutions

or alternative sets of substitutions that would require fewer nucleotide mutations than those of the Herfst *et al.* or Imai *et al.* sets. This work will be important for calculating risk and for monitoring in surveillance.

Fourth, further studies are needed to elucidate the changes in within-host fitness and between-host transmissibility associated with each respiratory droplet transmission-enabling substitution and combination of substitutions. These studies are necessary for determining the dynamics of within-host selection [including data on, and modeling of, the effects of glycan heterogeneity between the upper and lower respiratory tract (6)] and the potential for transmission of partially adapted viruses. It is important to determine the strength of selection at transmission because it can increase the proportion of respiratory droplet transmission-enabling substitutions. Further work is needed to refine the estimate for virus excretion and the minimum human infectious dose (29).

Numerous avian A/H5N1 viruses have been sampled in the past 2 years that are four nucleotide mutations from acquiring the Herfst *et al.* set of HA and PB2 substitutions and three nucleotide mutations from acquiring the Imai *et al.* set in HA (the Imai *et al.* set also requires a reassortment event). Precise estimates of the probability of evolving the remaining mutations for the virus to become a respiratory droplet-transmissible A/H5N1 virus cannot be accurately calculated at this time because of gaps in knowledge of the factors described above. However, the analyses here, using current best estimates, indicate that the remaining mutations could evolve within a single mammalian host, making the possibility of a respiratory droplet-transmissible A/H5N1 virus evolving in nature a potentially serious threat.

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Acknowledgments: C.A.R. was supported by a University Research Fellowship from the Royal Society. The authors acknowledge an Nederlandse Organisatie voor Wetenschappelijk Onderzoek (NWO) VICI grant, European Union (EU) FP7 programs EMPIRE (223498) and ANTIGONE (278976), Human Frontier Science Program (HFSP) program grant P0050/2008, Wellcome 087982AIA, the Bill and Melinda Gates Foundation (OPPGH5383), and NIH Director's Pioneer Award DP1-OD000490-01. R.A.M.F. was supported by National Institute of Allergy and Infectious Diseases (NIAID)–NIH contract HHSN266200700010C. A.E.X.B. was supported by a long-term fellowship from the HFSP. E.M., G.N., and Y.K. are supported by the Bill and Melinda Gates Foundation (OPPGH5383) and NIAID–NIH grant R01 AI 069274; in addition, Y.K. was supported by a Grant-in-Aid for Specially Promoted Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan and by ERATO. Y.K. and G.N. have a financial interest as founders of FluGen and hold a patent on influenza virus reverse genetics. Y.K. and G.N. have a paid consulting relationship with Theraclone; Y.K. also has a paid consulting relationship with Crucell. Y.K. has received speaker's honoraria from Chugai Pharmaceuticals, Novartis, Daiichi-Sankyo Pharmaceutical, Toyama Chemical, Wyeth, GlaxoSmithKline, and Astellas and grant support from Chugai Pharmaceuticals, Daiichi Sankyo Pharmaceutical, Toyama Chemical, Otsuka Pharmaceutical Company A.D.M.E.O. (on behalf of Viroclinics Biosciences BV) has advisory affiliations with GlaxoSmithKline, Novartis, and Roche. A.D.M.E.O. is Chief Scientific Officer of Viroclinics Biosciences BV. We thank S. Cornell, E. Ghedin, R. Johnstone, L. Reperant, and D. M. Smith for helpful discussions and the reviewers for their detailed and thoughtful comments.

Supplementary Materials

www.sciencemag.org/cgi/content/full/336/6088/1541/DC1
Materials and Methods
Figs. S1 to S15
Tables S1 to S5
References (39–66)

28 March 2012; accepted 31 May 2012
10.1126/science.1222526

A large, detailed portrait of Sir Isaac Newton, showing him with long, curly hair and a serious expression, wearing a dark coat over a white shirt.

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Wnt/ β -Catenin Signaling Regulates Telomerase in Stem Cells and Cancer Cells

Katrin Hoffmeyer, Angelo Raggioli, Stefan Rudloff, Roman Anton,* Andreas Hierholzer, Ignacio Del Valle, Kerstin Hein, Riana Vogt, Rolf Kemler†

Telomerase activity controls telomere length and plays a pivotal role in stem cells, aging, and cancer. Here, we report a molecular link between Wnt/ β -catenin signaling and the expression of the telomerase subunit *Tert*. β -Catenin-deficient mouse embryonic stem (ES) cells have short telomeres; conversely, ES cell expressing an activated form of β -catenin (β -cat ^{Δ Ex3/+}) have long telomeres. We show that β -catenin regulates *Tert* expression through the interaction with Klf4, a core component of the pluripotency transcriptional network. β -Catenin binds to the *Tert* promoter in a mouse intestinal tumor model and in human carcinoma cells. We uncover a previously unknown link between the stem cell and oncogenic potential whereby β -catenin regulates *Tert* expression, and thereby telomere length, which could be critical in human regenerative therapy and cancer.

Telomeres are specialized genomic structures that cap linear chromosomes and are essential for genome stability (1). Telomere length is controlled by the telomerase complex comprising an enzymatic subunit, TERT, and a RNA component, Terc (2). Embryonic and other stem cells have long telomeres, which become shorter during differentiation or aging but are stabilized again in tumorigenesis (3). The canonical Wnt signaling pathway plays a major role in regulating pluripotency in embryonic stem (ES) and adult stem cells from various tissues (4). β -Catenin is a central component of the Wnt pathway and forms a complex with members of the TCF family of transcription factors in the nucleus to control the transcription of target genes. Dysregulation of this pathway is frequently observed in human cancer (5). Here we show that TERT is directly regulated by β -catenin. Our results underline the cooperation between Wnt/ β -catenin signaling and telomerase in the control of stem cell renewal.

β -Catenin regulates *Tert* expression in mouse ES cells. Comparing the expression profiles of wild-type and β -catenin-deficient (β -cat^{-/-}) ES cells, we found that *Tert*, but not *Terc*, mRNA was significantly reduced in the absence of β -catenin (Fig. 1A and fig. S1A). ES cells harboring a stabilized active form of β -catenin (β -cat ^{Δ Ex3/+}) had higher levels of *Tert* mRNA compared to wild-type cells (Fig. 1A). Those differences in *Tert* mRNA expression were reflected by TERT protein amounts in Western blot analy-

sis (fig. S1C). Thus, altered levels of β -catenin affect *Tert* expression and may lead to differences in telomerase activity. Concordantly, telomerase activity was significantly increased in β -cat ^{Δ Ex3/+} compared to the wild type and was reduced in β -cat^{-/-} ES cells (Fig. 1B). Stimulation of wild-type ES cells with Wnt3a led to an increase in *Tert* expression (Fig. 1C). This supports recent findings that Wnt signaling may regulate *Tert* protein by sequestration of glycogen synthase kinase 3 (6). Knockdown of β -catenin by small interfering RNA (siRNA) reduced *Tert* expression and telomerase activity (Fig. 1D and fig. S1, D and E). Changes in *Tert* expression and telomerase activity resulted in different telomere lengths, with β -cat^{-/-} cells having shortened telomeres (Fig. 1E). Telomeres in wild-type ES cells were on average 50 kb long; in β -cat ^{Δ Ex3/+}, 75 kb; and in β -cat^{-/-} ES cells, 24 kb.

***Tert* is a direct target of β -catenin.** To gain insight into *Tert* transcriptional regulation by β -catenin, we performed luciferase reporter assays and chromatin immunoprecipitation (ChIP) experiments. *Tert* promoter fragments of 2.9 kb and 300 base pairs (bp) were equally active in wild-type ES cells (fig. S1F). The 300-bp fragment harbors binding sites for TCF and Klf4 (fig. S3C and S4G). β -Catenin was detected at the transcriptional start site (TSS) of *Tert* in wild-type ES cells by ChIP (Fig. 2A), which was further enhanced by the addition of Wnt3a (Fig. 2B). Binding of β -catenin to the TSS of *Tert* was also increased in β -cat ^{Δ Ex3/+} cells, whereas no β -catenin was immunoprecipitated in β -cat^{-/-} ES cells (Fig. 2A). The canonical β -catenin partners TCF3 and TCF4 were not detected at the *Tert* locus; however, TCF1 was enriched close to the TSS, even in β -cat^{-/-} cells (figs. S2, A and B, and S3A). We postulate that TCF1 may act as a transcriptional repressor of *Tert*, as knockdown of TCF1 by siRNA and mutational analysis of the 300-bp

promoter fragment suggest (fig. S3, B and D). In addition, no Oct3/4 binding at the *Tert* locus was observed (fig. S2C). c-Myc, a regulator of *Tert*, bound equally to the *Tert* promoter region in wild-type, β -cat^{-/-}, and β -cat ^{Δ Ex3/+} cells, although c-Myc protein was reduced in β -cat^{-/-} cells (fig. S2, D and E). Klf4 was of particular interest because of the conserved Klf4-binding site located at the TSS of the *Tert* promoter and because Klf4 contributes to the maintenance of telomerase activity in human cells (7). β -Catenin and Klf4 coimmunoprecipitate in wild-type ES cells (fig. S1G). Klf4 was identified at the *Tert* promoter by ChIP (Fig. 2C). Sequential ChIP revealed a Klf4/ β -catenin complex at the *Tert* promoter (Fig. 2D). To study the relationship between β -catenin and Klf4, we inhibited the expression of Klf4 in wild-type ES cells with siRNAs with or without Wnt3a stimulation (Fig. 2E and fig. S4). The accumulation of β -catenin on the *Tert* promoter upon Wnt3a stimulation was severely reduced after Klf4 knockdown. Therefore, Klf4 is required for β -catenin to localize to the *Tert* promoter. However, Klf4 alone was insufficient to drive *Tert* expression, as Klf4 was bound to the *Tert* promoter in β -cat^{-/-} cells, where *Tert* expression is low (Fig. 2C). Furthermore, whereas re-expression of β -catenin in β -cat^{-/-} cells led to an increase in *Tert* mRNA level, overexpression of Klf4 did not (Fig. 2F). Therefore, recruitment of β -catenin is necessary for *Tert* transcription in mouse ES cells, and Klf4 promotes this binding.

β -Catenin regulates *Tert* promoter activity. Supporting the role of β -catenin in *Tert* promoter activation, RNA polymerase II (Pol II), Pol II Ser5p (active Pol II), and the active trimethylated lysine-4 on histone-3 (H3K4me3) were detected at the *Tert* promoter in wild-type and β -cat ^{Δ Ex3/+}, but not in β -cat^{-/-} cells (Fig. 3, A and B, and fig. S5B). Both Pol II and H3K4me3 were reestablished at the *Tert* promoter in β -cat^{-/-} cells upon transfection with constitutively active β -catenin (Fig. 3C and fig. S6A). These results demonstrate that β -catenin is required for *Tert* promoter activation. We identified two members of the trithorax group (TrxG) proteins, Ash2l and Setd1a (8), the latter of which exhibited histone methyltransferase (HMT) activity at the TSS of the *Tert* promoter in ES cells. The localization of Ash2l and Setd1a at the *Tert* promoter depended on β -catenin, was not detected in β -cat^{-/-} ES cells, and was enhanced in β -cat ^{Δ Ex3/+} ES cells (Fig. 3, E and F, and figs. S5 and 6). Transfection experiments in human embryonic kidney 293 (HEK293) cells followed by coimmunoprecipitation revealed an association of β -catenin with Ash2l and Setd1a (Fig. 3D). These data suggest that β -catenin actively recruits HMTs to regulate the chromatin modifications required for the initiation of *Tert* transcription.

β -Catenin binds to the *Tert* promoter in adult stem cells. Next, we examined β -catenin at the *Tert* promoter in adult stem cells. The crypt of

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the small intestine contains stem cells; the villus contains more differentiated epithelial cells (9). β -Catenin bound to the TSS of *Tert* was only detected in the crypt cell fraction (Fig. 4A). This was further validated by β -catenin ChIP from isolated Lgr5-positive stem cells using Lgr5::GFP (green fluorescent protein) reporter mice (Fig. 4B) and in organoids from crypt cell cultures (fig. S7D). β -Catenin binding to the *Tert* promoter

(Fig. 4A) correlated with Pol II binding (fig. S9A) and different expression levels of *Tert* in the crypt versus villus fractions (Fig. 5B).

To determine whether our findings were relevant in other stem or progenitor cells, we made use of the Hes5::GFP reporter mouse (10) and analyzed primary neurospheres. In isolated Hes5::GFP neural stem cells, as well as in primary neurospheres, β -catenin was enriched at

the TSS of the *Tert* promoter (Fig. 4C). Deletion of β -catenin by adeno-mediated cre resulted in a reduction in *Tert* mRNA expression and abolished β -catenin binding to the *Tert* promoter (Fig. 4C and fig. S10).

β -Catenin regulates *Tert* expression in human cancer cells. Aberrant nuclear activation of β -catenin in cells of the villus leads to increased cell proliferation and the formation of polyps

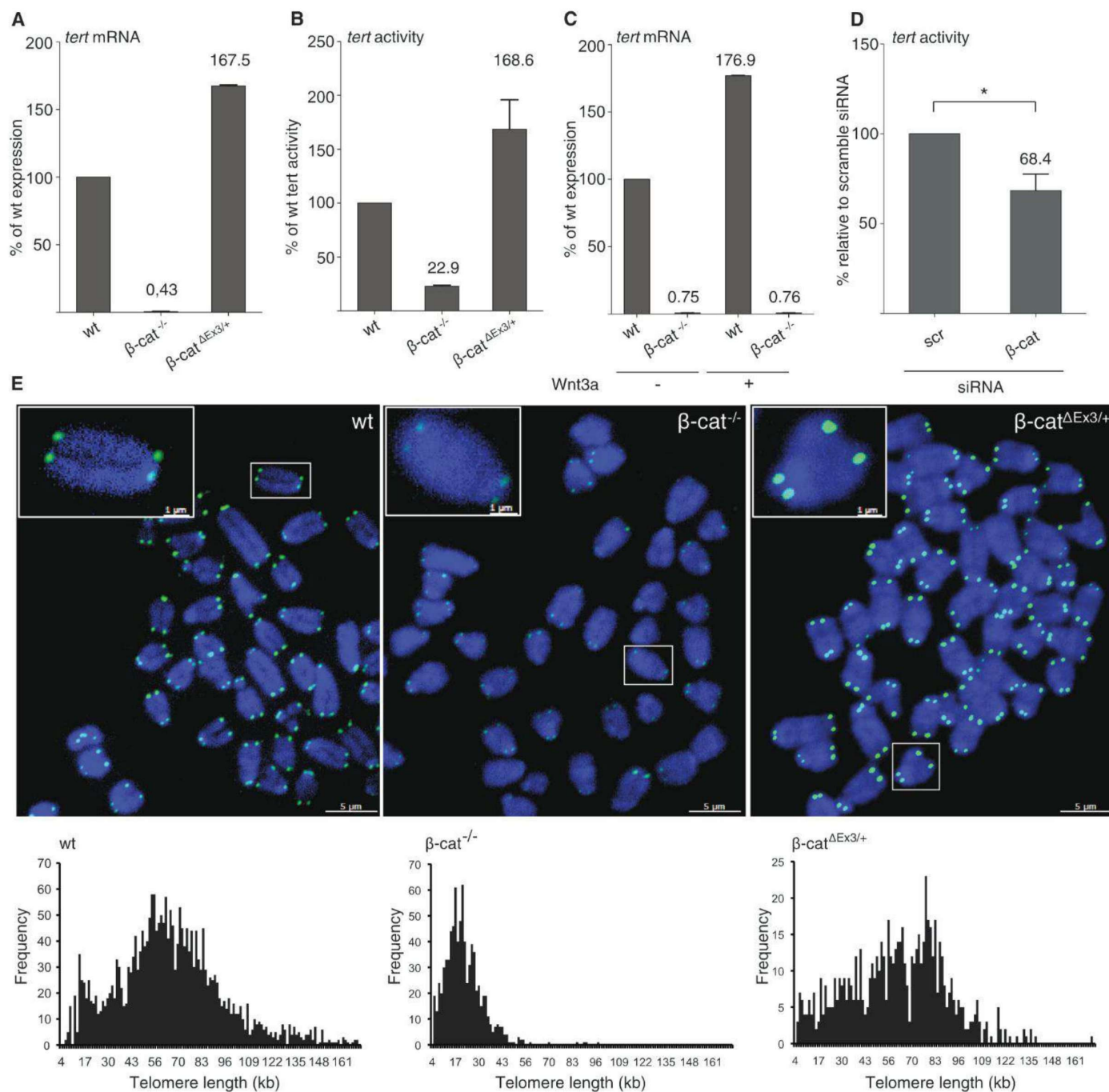


Fig. 1. β -Catenin regulates *Tert* mRNA expression, telomerase activity, and telomere length in genetically modified ES cells. (A) Quantitative polymerase chain reaction analysis of *Tert* mRNA. (B) Telomerase activity. (C and D) In wild-type (wt) ES cells Wnt3a induces *Tert* expression, whereas knockdown

of β -catenin by siRNA reduces *Tert* expression and activity (fig. S1). (E) Telomere length determined by quantitative fluorescence in situ hybridization analysis and TFL-Telo software in comparison to reference cell lines. ($n = 7$, $*P < 0.05$).

and adenomatous lesions (11). We established a mouse model to analyze *Tert* in hyperplastic lesions in the small intestine by the conditional activation of one β -catenin gain-of-function allele (villin-creERT $\times$ β -cat^{Ex3fl/+}). Stabilized β -catenin was detected in β -cat^{Ex3fl/+} crypt and villus cells (Fig. 5A). In the β -cat^{Ex3fl/+} intestine, hyperproliferative cells and induced expression of β -catenin target genes, such as those encoding c-Myc, Axin2, and CD44, were detected in the epithelia all

along the crypt-villus axis (Fig. 5A and fig. S8A). Expansion of the Paneth cell-specific marker, lysozyme, and CD44 was also observed in the villi (fig. S8B). Expression of *Tert* (Fig. 5B), *Lgr5*, and *Klf4* mRNAs (fig. S8A) was induced in the villus fraction of mutants. Concordantly, β -catenin and Pol II binding was increased at the TSS of *Tert* in villus cells (Fig. 5C and fig. S9B). These results provide strong in vivo evidence for the transcriptional regulation of *Tert* by β -catenin.

Finally, we examined whether the regulation of *Tert* expression by β -catenin may also be important in human cancer. We studied the human embryonal carcinoma cell line NTERA2 and the human colorectal carcinoma cell line SW480, the latter exhibiting increased amounts of cytoplasmic and nuclear β -catenin due to mutations in adenomatous polyposis coli (APC). β -Catenin was present at the TSS of *hTERT* (Fig. 5D), and knockdown of β -catenin by siRNA

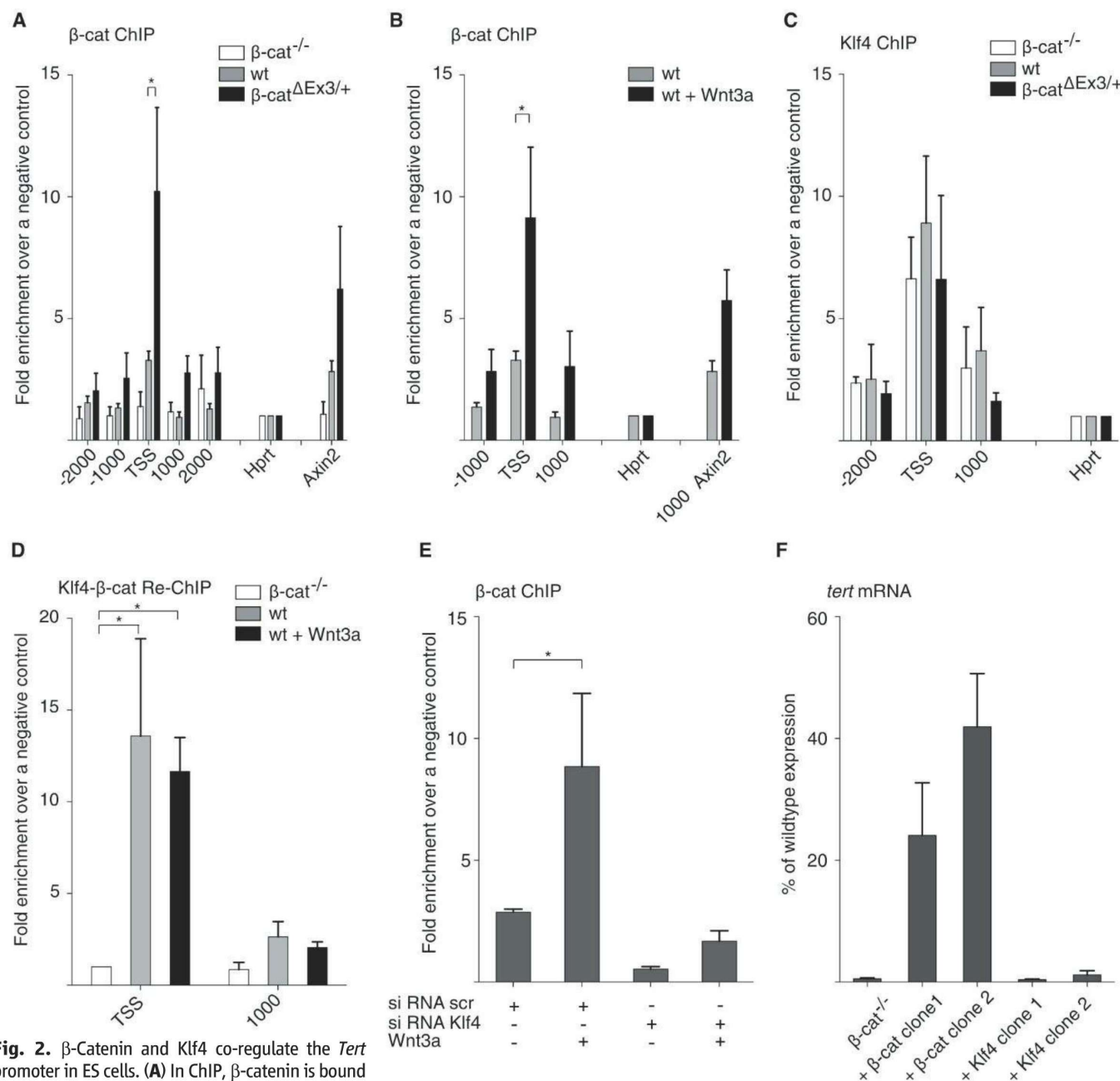


Fig. 2. β -Catenin and Klf4 co-regulate the *Tert* promoter in ES cells. (A) In ChIP, β -catenin is bound at the transcriptional start site (TSS) of *Tert* in wt and β -cat^{ΔEx3/+} but not in β -cat^{-/-} ES cells. (B) Enrichment of β -catenin at the TSS of *Tert* after stimulation with Wnt3a. (C) In ChIP, Klf4 is localized at the TSS of *Tert* in wt, β -cat^{ΔEx3/+}, and β -cat^{-/-} cells. (D) Re-ChIP, anti-Klf4 followed by anti- β -catenin, demonstrating that both form a complex at the *Tert* promoter. (E) Wild-type ES cells transfected with siRNA for Klf4 (48 hours) were cultivated with (+) or without (-) Wnt3a (12 hours). β -Catenin binding to the *Tert*

promoter was analyzed by ChIP (fig. S4). (F) β -cat^{-/-} cells were stably transfected with β -catenin or Klf4, and two independent clones were analyzed. Only overexpression of β -catenin resulted in reexpression of *Tert* mRNA. Axin2 was used as a positive control region and hypoxanthine phosphoribosyl-transferase (Hprt) as a negative control region in ChIP experiments. (* $P < 0.05$, table S3).

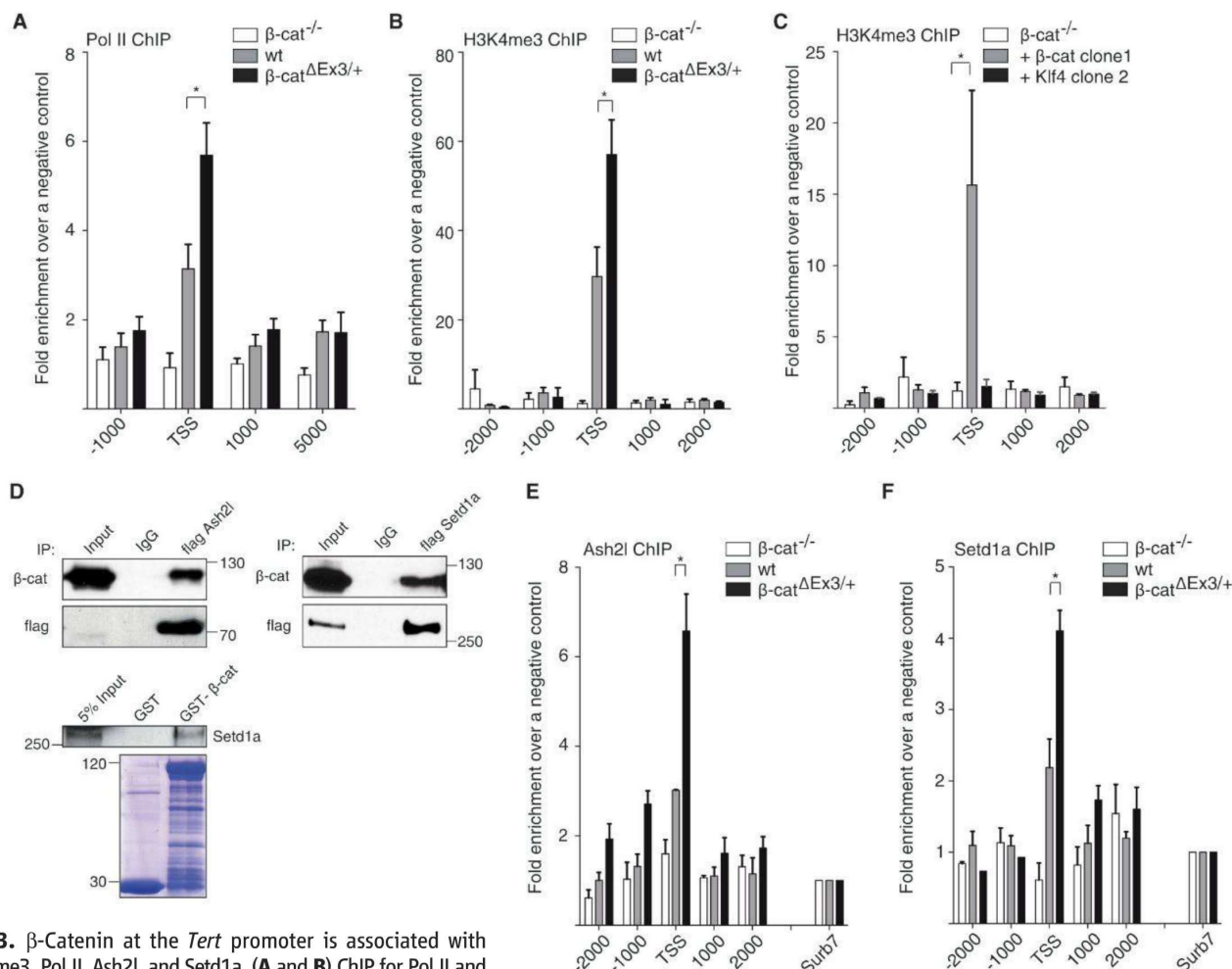


Fig. 3. β -Catenin at the *Tert* promoter is associated with H3K4me3, Pol II, Ash2l, and Setd1a. (**A** and **B**) ChIP for Pol II and H3K4me3 at the *Tert* promoter in wt, β -cat^{-/-}, and β -cat ^{Δ Ex3/+} ES cells (fig. S5). (**C**) ChIP for H3K4me3 and Pol II (fig. S6A) at the *Tert* promoter in β -cat^{-/-} cells overexpressing β -catenin or Klf4. (**D**) (Upper panels) β -Catenin and Setd1a (right) and β -catenin and Ash2l (left) association in HEK293 cells, as shown by coimmunoprecipitation (co-IP). In vitro-translated Setd1a

associates with glutathione *S*-transferase (GST)- β -catenin (lower panel). (**E** and **F**) Ash2l and Setd1a binding to the *Tert* promoter correlates with the β -catenin levels in wt, β -cat^{-/-}, and β -cat ^{Δ Ex3/+} ES cells as assessed by ChIP. Surb7, negative control. (**P* < 0.05, table S3).

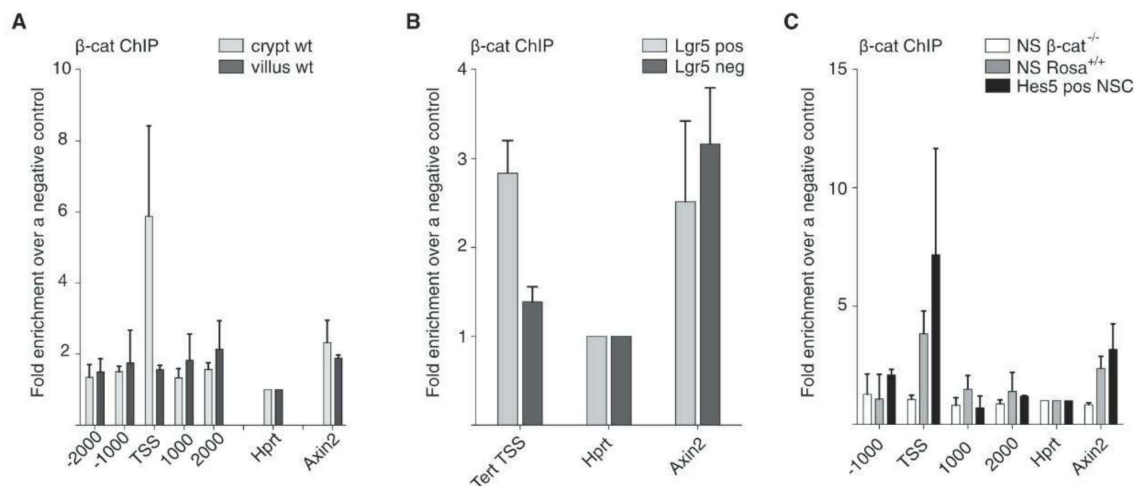


Fig. 4. Binding of β -Catenin at the *Tert* promoter in adult stem cells. (**A**) Mouse small intestines were separated into crypt and villus fractions and subjected to ChIP. β -Catenin at the TSS of *Tert* was detected only in the crypt cell compartment. (**B**) Lgr5-positive stem cells were isolated from Lgr5::GFP reporter mice by fluorescence-activated cell sorting (FACS) and subjected to

ChIP. (**C**) The Hes5::GFP reporter mouse was used to isolate neural stem cells by FACS. ChIP for β -catenin in isolated Hes5::GFP neural stem cells (NSC) and in neurospheres (NS) infected with adeno-cre revealed binding of β -catenin at the *Tert* promoter, which is abolished in neurospheres when β -catenin is deleted.

reduced *hTERT* mRNA levels in both cell lines (Fig. 5E and fig. S11C).

***Tert* is a β -catenin target.** By regulating *Tert* expression, β -catenin may assure the correct telomere length in stem cells, promoting their genomic stability and maintenance. Telomerase is directly involved in the regulation of Wnt/ β -catenin target genes (12). Our findings indicate a regulatory loop between β -catenin and *Tert* expression. In mouse ES cells, we identified Klf4 as a partner

of β -catenin in the regulation of *Tert* expression, but TCF1 may also be involved. The transcriptional regulation of *Tert* is very likely complex and combinatorial, and β -catenin may regulate *Tert* expression in other stem cell compartments in concert with other transcription factors. Clearly, in the absence of β -catenin, the *Tert* gene is silenced, but *Tert* transcription is initiated when β -catenin is recruited to the *Tert* promoter. We show that the initiation of *Tert* transcription is

accompanied by H3K4 trimethylation at the promoter, and we identified the lysine methyltransferase Setd1a as an interaction partner of β -catenin. Our results suggest that β -catenin recruits HMTs to initiate *Tert* transcription, which supports the increasingly recognized role of β -catenin in chromatin remodeling (13).

Furthermore, we show the localization of β -catenin at the *Tert* promoter to adult mouse stem cells and to human cancer cell lines, supporting

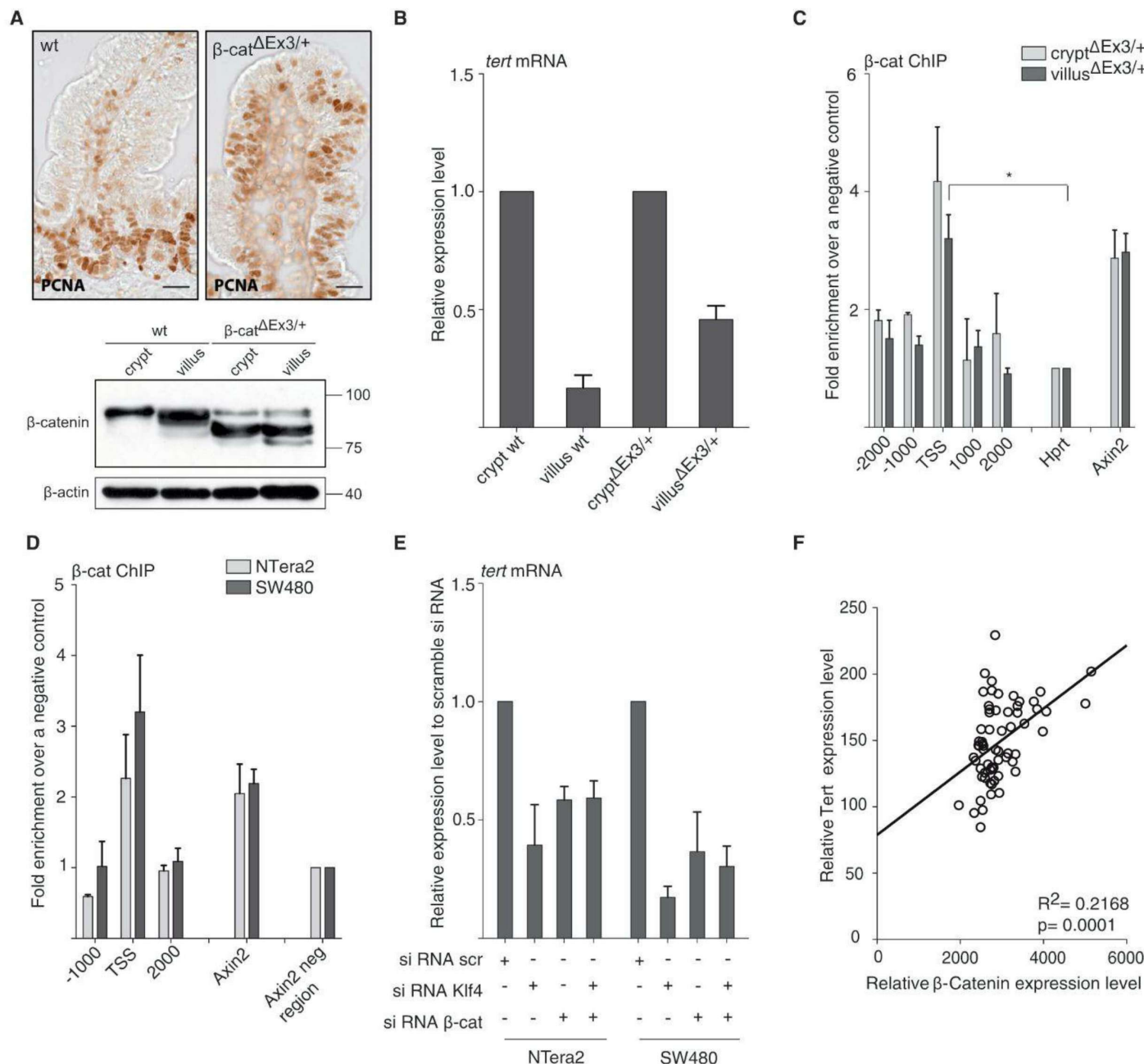


Fig. 5. β -Catenin regulates *Tert* in hyperproliferative intestinal epithelial cells and in human cancer cell lines. **(A)** Hyperplastic lesions in the intestine were induced by the stabilized active form of β -catenin (Villin-cre ERT $\times$ β -cat $^{\Delta Ex3/+}$), which resulted in hyperproliferative cells all along the crypt-villus axis, as seen by staining for proliferating cell nuclear antigen (PCNA) (upper panels). The stabilized form of β -catenin (β -cat $^{\Delta Ex3/+}$) is smaller in size and equally expressed in the crypt and villus fractions (lower panels). **(B)** Comparison of *Tert* mRNA

between crypt and villus compartments of wt and β -cat $^{\Delta Ex3/+}$ intestines. **(C)** In β -cat $^{\Delta Ex3/+}$ intestines, β -catenin is bound to the TSS of *Tert* in crypt and villus cell compartments (* $P < 0.05$, table S3). **(D)** In human NTERA2 and SW480 cells, β -catenin is bound at the TSS of *hTERT*, as detected by ChIP. **(E)** siRNA-mediated knockdown of β -catenin and Klf4 reduces expression of *TERT* mRNA. **(F)** Significant correlation between *TERT* and β -catenin expression in human colon cancer samples; $P < 0.0001$, $R^2 = 0.21068$ (for details, see fig. S11A).

the notion that the regulation of *Tert* by β -catenin is a general biological feature. Our mouse model (villin-creERT $\times$ β -cat^{Ex3fl/+}) provides in vivo evidence that the aberrant activation of β -catenin in the epithelium of the small intestine leads to *Tert* expression and binding of β -catenin to the *Tert* promoter. Exon 3 encodes phosphorylation sites important for β -catenin protein stability (14), and these sites are frequently mutated in human colorectal and other cancers (15). It is notable that in human colorectal cancers, high *Tert* and β -catenin expression are significantly correlated ($P=0.0001$), as taken from publicly available microarray data sets (16) (Fig. 5F and fig. S11A).

Conclusion. By identifying *Tert* as a target gene of β -catenin, we demonstrate a link between these two key regulators in stem cell biology and cancer. From the results presented here, we propose that mutations in β -catenin can lead to an enhanced *Tert* expression in human cancer, which

results in the stabilization of telomeres, one of the hallmarks of tumorigenesis.

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Acknowledgments: We thank H. H. Ng (Singapore) for the antibody against Klf4, I. Horikawa (NIH) for the 300-bp promoter construct, and J.-H. Lee (Indiana University) for Ash2L and Setd1a expression vectors. We thank E. M. Varela and M. Blasco (Madrid) for advice and reagents for the Q-fish analysis and for antibodies against Tert. We are grateful to M. M. Taketo (Kyoto) and to S. Robine (Paris) for providing mutant mice. We thank S. Lugert for advice on neurosphere cultures, J. Volkind for technical assistance, V. Taylor and D. Junghans for discussion and critical reading, and R. Schneider for typing of the manuscript. K.H. and A.R. are members of the International Max Planck Research School for Molecular and Cellular Biology (IMPRS-MCB). This work was supported by the Max Planck Society. The authors declare no financial interests.

Supplementary Materials

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22 December 2011; accepted 9 April 2012
10.1126/science.1218370

REPORTS

A Sharp Peak of the Zero-Temperature Penetration Depth at Optimal Composition in BaFe₂(As_{1-x}P_x)₂

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In a superconductor, the ratio of the carrier density, n , to its effective mass, m^* , is a fundamental property directly reflecting the length scale of the superfluid flow, the London penetration depth, λ_L . In two-dimensional systems, this ratio n/m^* ($\sim 1/\lambda_L^2$) determines the effective Fermi temperature, T_F . We report a sharp peak in the x -dependence of λ_L at zero temperature in clean samples of BaFe₂(As_{1-x}P_x)₂ at the optimum composition $x = 0.30$, where the superconducting transition temperature T_c reaches a maximum of 30 kelvin. This structure may arise from quantum fluctuations associated with a quantum critical point. The ratio of T_c/T_F at $x = 0.30$ is enhanced, implying a possible crossover toward the Bose-Einstein condensate limit driven by quantum criticality.

In two families of high-temperature superconductors, cuprates and iron-pnictides, superconductivity emerges in close proximity to an antiferromagnetically ordered state, and the critical temperature, T_c , has a dome-shaped dependence on doping or pressure (1–3). What happens inside this superconducting dome is still a matter of debate (3–5). In particular, elucidating whether a quantum critical point (QCP) is hidden inside it (Fig. 1, A and B) may be key to understanding high- T_c superconductivity (5, 6). A QCP marks the position of a quantum phase transition (QPT), a zero-temperature phase transition driven by quantum fluctuations (7).

The London penetration depth, λ_L , is a property that may be measured at low temperature in the superconducting state to probe the elec-

tronic structure of the material and to look for signatures of a QCP. The absolute value of λ_L in the zero-temperature limit immediately gives the superfluid density $\lambda_L^{-2}(0) = \mu_0 e^2 \Sigma_i n_i / m_i^*$, which is a direct probe of the superconducting state; here, m_i^* and n_i are the effective mass and concentration of the superconducting carriers in band i , respectively (8). Measurements on high-quality crystals are necessary because impurities and inhomogeneity may otherwise wipe out the signatures of the QPT. Another advantage of this approach is that it does not require the application of a strong magnetic field, which may induce a different QCP or shift the zero-field QCP (9).

BaFe₂(As_{1-x}P_x)₂ is a particularly suitable system for penetration depth measurements be-

cause, in contrast to most other Fe-based superconductors, very clean (10) and homogeneous crystals of the whole composition series can be grown (11). In this system, the isovalent substitution of P for As in the parent compound BaFe₂As₂ offers an elegant way to suppress magnetism and induce superconductivity (11). Non-Fermi liquid properties are apparent in the normal state above the superconducting dome (Fig. 2A) (11, 12), and de Haas-van Alphen (dHvA) oscillations (10) have been observed over a wide x range, including the superconducting compositions, giving detailed information on the electronic structure. Because P and As are isoelectronic, the system remains compensated for all values of x (i.e., volumes of the electron and hole Fermi surfaces are equal).

As discussed in (10), the normal-state electronic structure of BaFe₂(As_{1-x}P_x)₂ determined by dHvA experiments is significantly modified from that predicted by conventional density functional theory (DFT) band structure calculations. Figure 2A shows the composition evolution of the effective mass, m^* , normalized by the free

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electron mass, m_0 , and the Fermi temperature, $T_F = \epsilon_F/k_B = \frac{\hbar^2}{2\pi k_B m^*} A_k$, for $x > 0.4$, determined from the dHvA oscillations corresponding to the extremal orbits on the outer electron Fermi surface (β_1 and β_2 orbits in Fig. 2B). Here, ϵ_F is the Fermi energy and A_k is the cross-sectional area of the

orbit. In contrast to the negligible x -dependence expected from the DFT calculations, a critical-like increase in m^* accompanied by a strong reduction of T_F is observed as the system is tuned toward the optimal composition from the overdoped side.

For a reliable determination of the absolute value of $\lambda_L(0)$ in small single crystals, we adopted

three different methods (8). The first is the lower- T_c superconducting film coating method (13–15), in which $\lambda_L(0)$ is determined from the frequency shift of a high-precision tunnel diode oscillator (16) (resonant frequency of $f \sim 13$ MHz) containing the $\text{BaFe}_2(\text{As}_{1-x}\text{P}_x)_2$ crystal coated with an aluminum film ($T_c = 1.2$ K) of known thickness and penetration depth.

The second is the microwave cavity perturbation technique, in which $\lambda_L(0)$ is determined from the measurements of surface impedance, $Z_s = R_s + iX_s$, by using a superconducting resonator ($f \sim 28$ GHz) and a rutile cavity resonator ($f \sim 5$ GHz), both of which have a very high quality factor $Q \sim 10^6$ (8). In all crystals, the residual surface resistance $R_s(0)$ at $T \rightarrow 0$ K, which we determined by withdrawing the crystal from the rutile cavity at low temperature, is less than 0.3% of R_s just above T_c . This negligible residual $R_s(0)$ indicates almost perfect Meissner screening without any non-superconducting regions. In the superconducting state well below T_c , $\lambda_L(T)$ is obtained from the surface reactance via the relation $X_s(T) = \mu_0 \omega \lambda_L(T)$. The absolute value of X_s is determined from Z_s and dc-resistivity ρ_{dc} (measured separately by a conventional four contact technique) by the relation $R_s = X_s = \sqrt{\mu_0 \omega \rho_{dc} / 2}$ which holds in the normal state (8).

The third method uses the temperature-dependent changes $\delta\lambda_L(T) = \lambda_L(T) - \lambda_L(0)$, measured by the tunnel diode oscillator down

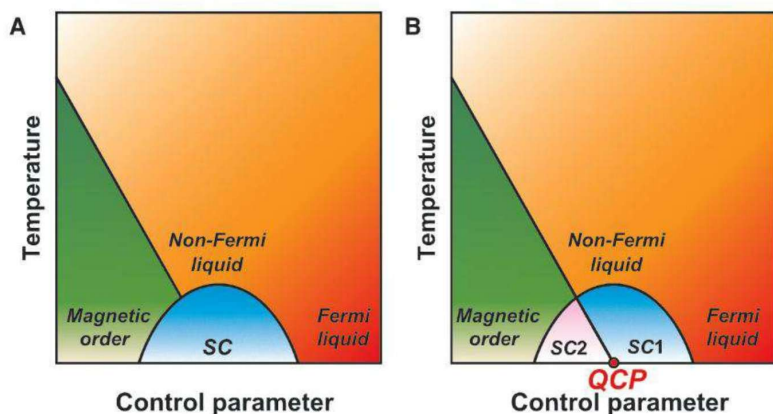
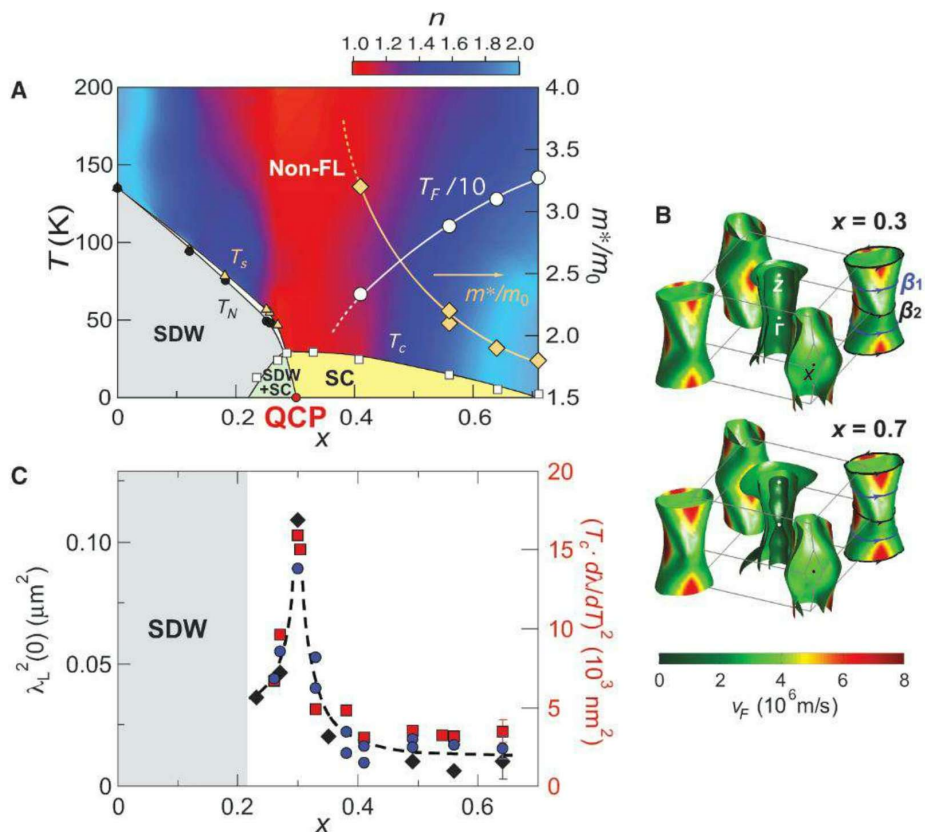


Fig. 1. Generic temperature versus nonthermal control parameter phase diagram of iron-based superconductors, illustrating two cases. (A) Quantum criticality is avoided by the transition to the superconducting state. There is only one superconducting phase. (B) A QCP lies beneath the superconducting dome. The QCP separates two distinct superconducting phases (SC1 and SC2). In the case of (A), non-Fermi liquid behavior may appear above the dome if there is a QCP located along the axis of another control parameter that is independent of the control parameter shown on the abscissa. In the case of (B), non-Fermi liquid behavior appears because of the QCP inside the dome.

Fig. 2. (A) Phase diagram of $\text{BaFe}_2(\text{As}_{1-x}\text{P}_x)_2$. The transition to the SDW ground state at T_N coincides with or is preceded by the structural transition at T_s . With increasing x , T_N decreases and goes to zero continuously at $x = 0.30$. The superconducting dome extends over a composition range $0.22 < x < 0.7$, with maximum $T_c = 30$ K at $x = 0.30$. The red shaded region at around $x = 0.30$ represents the region where the exponent n of the temperature dependence of the resistivity, $\rho_{dc}(T) = \rho(0) + aT^n$, is close to unity, which is a hallmark of a non-Fermi liquid (non-FL). The composition dependence of the effective Fermi temperature T_F and renormalized mass m^*/m_0 determined by dHvA oscillations (10) arising from the β orbits [shown in (B)] are also plotted. (B) Fermi surface of $\text{BaFe}_2(\text{As}_{1-x}\text{P}_x)_2$ with $x = 0.3$ and 0.7 from the band-structure calculation using DFT as implemented in the WIEN2K code (10). The Fermi surface consists of five quasi-cylindrical pockets, three hole pockets at the center of the Brillouin zone, and two electron pockets centered at its corners. The shading represents the in-plane Fermi velocity, v_F . The flat parts of the outer electron sheets have high v_F values. The lines represent the extremal β orbits. (C) Composition evolution of the square of the London penetration depth $\lambda_L^2(0)$ in the zero-temperature limit determined by three different methods: aluminum coating method (black diamonds), microwave cavity perturbation technique (blue circles), and the low-temperature slope of the change of the penetration depth with temperature (red squares, right-hand scale) shown in Fig. 3. Different points for the same x correspond to different crystals from the same batch, but for some of the microwave and the low-temperature slope data we used the same crystals. Error bars shown for $x = 0.64$ represent typical experimental errors (8) largely resulting from uncertainties in the determination of geometrical factors.



to ~ 80 mK (Fig. 3). For all samples measured, covering a wide range of concentrations $0.26 \leq x \leq 0.64$, a quasi- T -linear variation of $\delta\lambda_L(T)$ is observed. This important result indicates that the presence of line nodes in the superconducting gap (16) is a robust feature of this P-substituted system. This robustness is consistent with the nodes being on the electron sheets (17) rather than the hole sheets, because the electron sheets change relatively little with x whereas the shape of the hole sheets changes substantially (Fig. 2B). A notable feature of the T -linear penetration depth is that the relative slope $d\lambda_L/d(T/T_c)$ is steepest for $x = 0.30$ (Fig. 3B). In general, this slope is determined by the Fermi velocity and the k dependence of the superconducting gap close to the node. Making the reasonable assumption that the gap structure evolves weakly across the phase diagram, the x dependence of $d\lambda_L/d(T/T_c)$ will mirror that of $\lambda_L(0)$ (8).

Figure 2C shows the composition dependence of the squared in-plane London penetration length $\lambda_L^2(0)$ in the zero-temperature limit. Although different techniques can involve systematic errors (15), all three methods give very similar x dependencies. The most notable feature is the sharp peak in $\lambda_L^2(0)$ at $x = 0.30$, at about the same composition level where T_c is maximal. The prominent enhancement of $\lambda_L^2(0)$ is observed on approaching $x = 0.30$ from either side and has been seen in multiple samples by using different techniques. This reproducibility, combined with the above mentioned low $R_s(0)$, sharp superconducting transitions, and large heat capacity anomalies at all values of x close to $x = 0.30$ (8), shows that the enhancement is not an experimental artifact associated with poor screening caused by nonbulk superconductivity. We attribute the peak in $\lambda_L^2(0)$ to the existence of a QCP at $x = 0.30$.

This result contrasts with the behavior found for the electron-doped iron-based superconductors, $\text{Ba}(\text{Fe}_{1-x}\text{Co}_x)_2\text{As}_2$, where a shallow minimum of $\lambda_L(0)$ at the optimum doping and a continuous increase on the underdoped side have been reported (14, 15, 21). This difference from the present case may be related to a greater degree of electronic disorder in the Fe layer caused by the Co doping (1, 2), which may smear out the singularity. The difference in the superconducting gap structure (2) as well as the addition of charge carriers from the Co doping may also be a source of differences in the x -dependence of $\lambda_L(0)$.

In cuprates, a QCP associated with the pseudogap formation has been suggested to occur at the hole concentration $p \sim 0.19$ inside the superconducting dome. However, there does not appear to be any evidence of mass divergence at this purported QCP, and at this doping a broad minimum of $\lambda_L^2(0)$ was reported (22). An enhancement in $\lambda_L^2(0)$ has been observed at $p \sim 1/8$ (23), but this is accompanied by a reduction of T_c , which is again different from the present case where the peak in $\lambda_L^2(0)$ coincides with the maximum T_c .

Our results may have general implications for the behavior of $\lambda_L^2(0)$ in strongly correlated superconducting systems. How strong electron correlations influence the condensed electron pairs in superconductors has been a long-standing is-

sue (24–26). In fact, it has been pointed out that, in an ordinary one-component Galilean invariant Fermi liquid, electron correlation effects do not cause the renormalization of λ_L in the superconducting state (24). However, experimentally

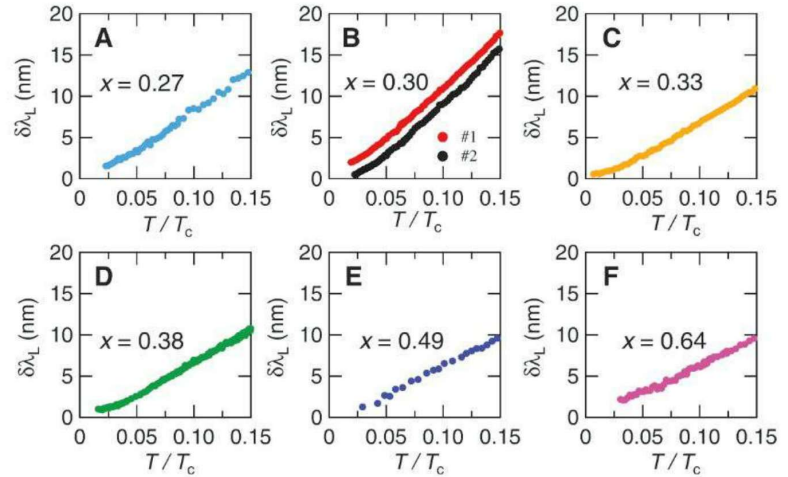


Fig. 3. Relative change of the penetration depth, $\delta\lambda_L(T) = \lambda_L(T) - \lambda_L(0)$, at low temperatures plotted against T/T_c for different compositions from $x = 0.27$ (A) to 0.64 (F). For $x = 0.30$, data for two samples are shown, one of which (#2) is shifted vertically for clarity.

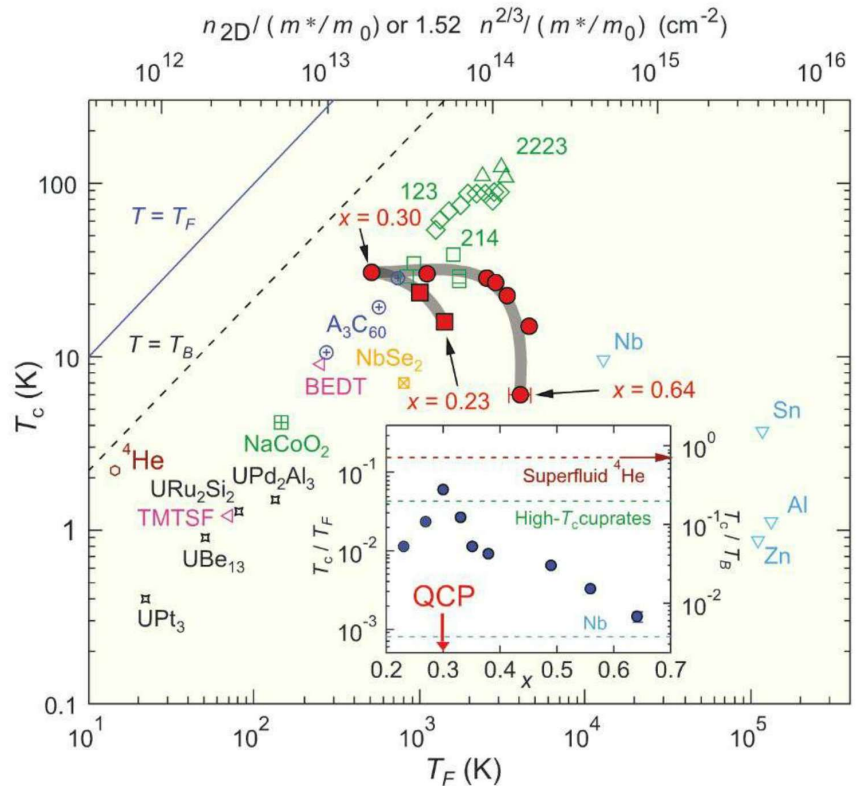


Fig. 4. Uemura plot. T_c is plotted as a function of T_F evaluated from $1/\lambda_L^2(0)$ for various superconductors [$n_{2D}/(m^*/m_0)$ for 2D and $1.52n^{2/3}/(m^*/m_0)$ for 3D systems] (27). We used an average of the Al-coating and microwave data for $\text{BaFe}_2(\text{As}_{1-x}\text{P}_x)_2$. The data for $x \geq 0.30$ (red circles) and for $x < 0.30$ (red squares) bridge a gap between the conventional superconductors such as Nb and cuprate high- T_c superconductors such as $(\text{La},\text{Sr})_2\text{CuO}_4$ (214), $\text{YBa}_2\text{Cu}_3\text{O}_{7-\delta}$ (123), and $\text{Bi}_2\text{Sr}_2\text{Ca}_2\text{Cu}_3\text{O}_y$ (2223). $x = 0.30$ represents the data at the QCP. The dashed line is the BEC temperature for the ideal 3D boson gas. (Inset) Composition dependence of T_c normalized by the Fermi temperature (left axis) or BEC temperature (right axis). Green and light blue dashed lines mark the T_c/T_F values for underdoped cuprates 123 and for the conventional superconductor Nb. Brown arrow represents $T_c/T_B = 0.7$ for superfluid ^4He .

$\lambda_L^{-2}(0)$ does appear to be enhanced in heavy-fermion superconductors, which contain interacting conduction electrons and local moments (25, 26). The present results in $\text{BaFe}_2(\text{As}_{1-x}\text{P}_x)_2$ support this and suggest that in sufficiently clean systems electron correlation effects can lead to a striking renormalization of $\lambda_L^{-2}(0)$.

We now discuss the consequences of a QPT inside the superconducting dome. Such a QPT implies that the non-Fermi liquid behavior indicated by the red region in Fig. 2A is most likely associated with a finite temperature quantum critical region linked to the QCP. Moreover, this transition immediately indicates two distinct superconducting ground states. In our system, the robust T -linear behavior of $\delta\lambda_L(T)$ on both sides of the purported QCP at $x = 0.30$ argues against a drastic change in the superconducting gap structure (2, 6). The fact that the zero-temperature extrapolation of the antiferromagnetic transition $T_N(x)$ into the dome (12) coincides with the location of the QCP (Fig. 2A) may indicate that the QCP separates a pure superconducting phase on the right and a superconducting phase coexisting with spin-density-wave (SDW) order on the left (Fig. 1B).

To place $\text{BaFe}_2(\text{As}_{1-x}\text{P}_x)_2$ in the context of other superconductors, we plotted T_c as a function of the effective Fermi temperature T_F for several types of compounds (Uemura plot, Fig. 4); the red symbols correspond to various values of x for $\text{BaFe}_2(\text{As}_{1-x}\text{P}_x)_2$ studied here, and the others are obtained from μSR measurements reported previously (27). Because the relevant Fermi surface sheets are nearly cylindrical, T_F for two-dimensional (2D) systems may be estimated directly from $\lambda_L(0)$ via the relation $T_F = \frac{(\hbar^2\pi)n_{2D}}{k_B m^*} \approx \left(\frac{\hbar^2\pi}{\mu_0 e^2 d}\right) \lambda_L^{-2}(0)$, where n_{2D}

is the carrier concentration within the superconducting planes and d is the interlayer spacing; $T_F = (\hbar^2/2)(3\pi^2)^{2/3} n^{2/3}/k_B m^*$ for 3D systems (27). The dashed line corresponds to the Bose-Einstein condensation (BEC) temperature for an ideal 3D boson gas, $T_B = \frac{\hbar^2}{2\pi m^* k_B} \left(\frac{n}{2.612}\right)^{2/3}$. In a quasi-2D system, this value of T_B provides an estimate of the maximum condensate temperature. The evolution of T_c with T_F in the present system is in sharp contrast to that in cuprates, in which T_c is roughly scaled by T_F . The inset of Fig. 4 depicts the x -composition dependence of T_c normalized by Fermi (or BEC) temperature, T_c/T_F (T_c/T_B). In the large composition region ($x > 0.6$), T_c/T_F is very small, comparable to that of the conventional superconductor Nb. As x is decreased, T_c/T_F increases rapidly and then decreases in the SDW region after reaching the maximum at the QCP ($x = 0.30$). Notably, the magnitude of T_c/T_B (≈ 0.30) at the QCP exceeds that of cuprates and reaches almost 40% of the value of superfluid ^4He .

The fact that T_c/T_F becomes largest at the QCP indicates that the strongest pairing interaction is achieved at the QCP, implying that

high- T_c superconductivity is driven by the QCP. In a multiband system, we need to introduce the effective Fermi energy ϵ_F for each band, which is defined for electron bands as the energy of the highest occupied state relative to the top of the band and for hole bands as the energy of the highest occupied state relative to the bottom of the band. Because the outer electron sheet with the highest Fermi velocity has the largest ϵ_F and hence the largest contribution to $\lambda_L^{-2}(0)$, the magnitudes of T_c/T_F in the other sheets are expected to be even larger. These results lead us to consider that in terms of T_c/T_F the system is closer to the Bardeen-Cooper-Schrieffer-BEC crossover (28–30) than the cuprates.

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Acknowledgments: We thank R. M. Fernandes, S. Kivelson, H. Kontani, Q. Si, U. Welp, and Y. Yanase for valuable discussion. Supported by Grant-in-Aid for the Global Centers of Excellence program "The Next Generation of Physics, Spun from Universality and Emergence," Grant-in-Aid for Scientific Research on Innovative Areas "Heavy Electrons" from the Ministry of Education, Culture, Sports, Science and Technology of Japan, KAKENHI from Japan Society for the Promotion of Science, and the Engineering and Physical Sciences Research Council (UK). Work at the Ames Laboratory was supported by the U.S. Department of Energy, Basic Energy Sciences, Materials Science and Engineering Division, under contract no. DE-AC02-07CH11358. Work at the University of Illinois was supported by the Center for Emergent Superconductivity, an Energy Frontier Research Center funded by the U.S. Department of Energy, Office of Science, Office of Basic Energy Sciences under award no. DE-AC0298CH1088.

Supplementary Materials

www.sciencemag.org/cgi/content/full/336/6088/1554/DC1
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30 January 2012; accepted 10 May 2012
10.1126/science.1219821

Electromechanical Properties of Graphene Drumheads

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We determined the electromechanical properties of a suspended graphene layer by scanning tunneling microscopy (STM) and scanning tunneling spectroscopy (STS) measurements, as well as computational simulations of the graphene-membrane mechanics and morphology. A graphene membrane was continuously deformed by controlling the competing interactions with a STM probe tip and the electric field from a back-gate electrode. The probe tip-induced deformation created a localized strain field in the graphene lattice. STS measurements on the deformed suspended graphene display an electronic spectrum completely different from that of graphene supported by a substrate. The spectrum indicates the formation of a spatially confined quantum dot, in agreement with recent predictions of confinement by strain-induced pseudomagnetic fields.

Suspending graphene sheets can remove unwanted electrical potential disturbances from supporting substrates. Initial measurements of graphene devices on SiO_2 insulating substrates achieved carrier mobilities of $\approx 5000 \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}$ (*I*). Removing the substrate by suspending graphene resulted in mobilities in excess of $200,000 \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}$ at low temperatures

(2). These differences illustrate how substrate-induced potential disorder due to impurities and strain can play a role in the electronic properties of graphene. Recently, strain engineering of the electronic properties of graphene, which can be described through the generation of local pseudoscalar and magnetic fields by strain (3–11), has attracted considerable attention. For example,

strain-generated pseudomagnetic fields equivalent to a real magnetic field as high as 300 T have been reported (5). However, a number of questions remain regarding the structure and electronic properties of suspended graphene layers.

In this Report, we present scanning tunneling microscopy (STM) and scanning tunneling spectroscopy (STS) measurements of suspended graphene drumheads in a back-gated graphene device structure. We achieved stable STM measurements on the suspended graphene by carefully approaching the membrane with very slow scanning speeds. We observed that both the van der Waals forces from the STM probe tip and electrostatic force induced by back-gate voltage can induce substantial mechanical deformation in the suspended graphene membranes. The visible membrane shape measured as a tip height can be continuously tuned from concave to convex by adjusting the electrostatic force. However, the induced strain in the graphene membrane mainly originates from the tip-membrane interaction, dramatically altering the electronic spectrum of graphene compared with the measurements of graphene directly supported by a substrate (12, 13). In particular, we observed multiple quartet bands of peaks in the differential conductance spectra that are characteristic of charge confinement in a quantum dot (QD). Theoretical simulations of the membrane mechanics and experimental results confirm recent predictions of QD confinement in pseudomagnetic fields generated by rotationally symmetric strain fields in graphene membranes (6, 7).

Figure 1, A to C, shows the geometry of the graphene device we used in this study. We fabricated an array of pits in SiO₂/Si substrates, 1.1 μm in diameter and 100 nm in depth, by shallow plasma etching of SiO₂ (Fig. 1C). Graphene flakes were exfoliated onto the prepatterned SiO₂/Si substrate via mechanical exfoliation of natural graphite and contacted using a Pd/Au electrode deposited via a stencil mask (Fig. 1B). After fabrication, we aligned the STM probe tip onto the device in ultrahigh vacuum by using an external optical microscope before cooling the STM module in a custom STM system operating at 4 K (14).

STM topographic images of single-layer supported and suspended graphene over an area of 20 nm by 20 nm are shown in Fig. 1, D and E, respectively. On small length scales, the graphene honeycomb lattice was clearly resolved on both supported and suspended graphene, with compa-

parable corrugation amplitudes (Fig. 1, D and E, insets). On the 20-nm length scale, the peak-to-peak height corrugation on the suspended graphene was about four times larger than that of the supported graphene on the SiO₂ substrate over this scan area range (Fig. 1F). The larger height variation on the graphene membrane indicates deformation of the graphene sheet, which becomes larger with greater scan sizes (Fig. 2).

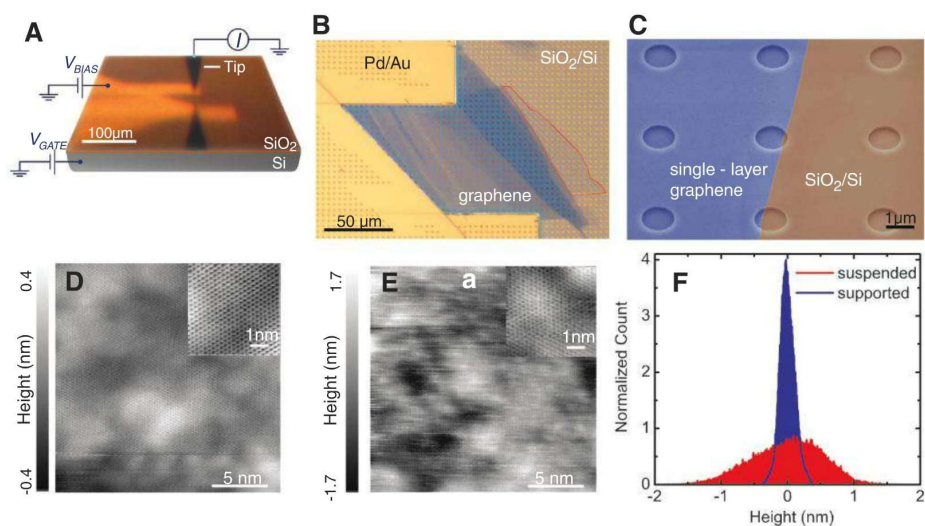


Fig. 1. STM measurements of graphene drumheads. (A) Optical image of the gated graphene device. The device consists of a single graphene layer placed over an array of pits (1.1 μm in diameter, 100 nm in depth) etched in SiO₂ (300 nm)/Si substrate. (B) Magnified optical image of the device in (A). The single-layer graphene region is denoted by a red outline. (C) Scanning electron microscope image of the device. STM topographic images, 20 by 20 nm, on the supported graphene (D) and suspended graphene membrane (E). Insets show the graphene atomic lattice images (5 by 5 nm). The inset gray scales cover the range of ± 0.2 nm. (F) Topographic height histograms from the images in (D) and (E).

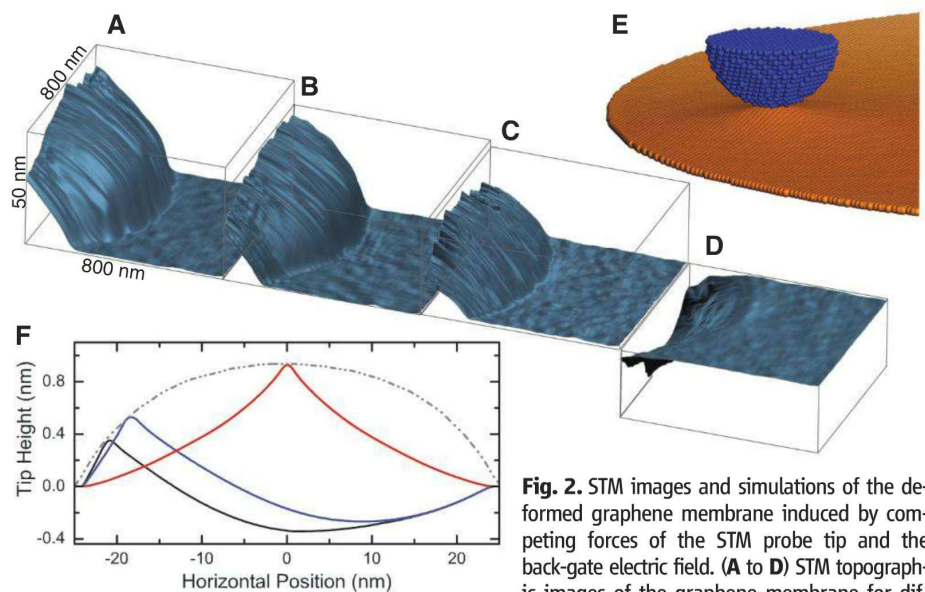


Fig. 2. STM images and simulations of the deformed graphene membrane induced by competing forces of the STM probe tip and the back-gate electric field. (A to D) STM topographic images of the graphene membrane for different gate voltages: 0 V (A), 20 V (B), 40 V (C), and 60 V (D). The membrane is deformed by upward forces from the STM tip and a downward force due to the electric field from the applied V_{GATE} . (E) Atomistic model showing deformations in the graphene membrane interacting with the STM tip. The radii of the tip and membrane in this model are 2.5 and 25 nm, respectively. (F) Calculated membrane shapes at the critical tip height for horizontal tip positions of 2.5 nm (black curve), 5.2 nm (blue curve), and 25 nm (red curve) from the membrane edge. A back-gate force of 0.012 pN was applied to each atom in the membrane. The gray dot-dashed curve is an envelope showing the membrane height as seen in a STM measurement.

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be observed as the tip scanned the graphene suspended over the pits in the substrate (Fig. 2, A to D). This visible deformation could be continuously tuned to be either positive (outward from the surface, Fig. 2, A to C) or negative (inward to the surface, Fig. 2D), depending on the magnitude of the voltage, V_{GATE} , applied to the back-gate electrode.

The graphene deformation in Fig. 2, A to D, was caused by the STM probe tip pulling up on the membrane via the van der Waals and electrostatic forces and the back-gate electrode pulling the membrane down via electrostatic force. In the progression of images from Fig. 2, A to D, the force from the back gate was incrementally increased as V_{GATE} was changed from 0 to 60 V, which progressively pulled the membrane downward. Between 50 and 60 V, the forces from the probe tip were balanced by the gate field, and finally at 60 V, the force from the back gate pulled the graphene into the underlying pit. In contrast, a tip-membrane potential difference in the range of ± 1 V and the related electrostatic force produced only a small effect on the membrane

shape, implying that the van der Waals force between the probe tip and graphene membrane is the dominant pull-up force. We also augmented our spectroscopic measurements with molecular dynamics simulations discussed below.

To study the electronic properties of the suspended graphene membranes, we applied gate-mapping STS measurements at high resolution in which individual dI/dV spectra (where I is current) are measured at a fixed spatial location as a function of both tunneling bias, V_B , and back-gate voltage, V_{GATE} (Fig. 3) (12, 15). The dI/dV spectra, proportional to the local density of states, were used to examine how the suspension of the graphene affects its electronic spectrum. The electronic spectrum of graphene in a uniform applied magnetic field, B , consists of a set of quantized Landau levels (LLs) (16, 17). Gate-mapping measurements made on the suspended membrane at the same fixed location (Fig. 3, A to D) show a graphene electronic spectrum dramatically different from the measurements on supported graphene layers (12, 13). The notable signatures of the spectra of supported graphene [see (12, 13)]

are the following: (i) In zero applied magnetic field, tunneling spectra show a minimum at the Dirac point with its distinct square-root dispersion as a function of density (18), and (ii) in applied magnetic fields, magnetically quantized LLs form a staircase pattern in the gate maps as the LLs are sequentially pinned at the Fermi level. Spectra are very different on the suspended membrane. In zero applied magnetic field, the dI/dV gate map (Fig. 3A) showed a new series of states seen as lines with a small negative slope with increasing gate voltage (almost horizontal across the gate map). In addition, we observed a series of broad bands with positive slopes, as indicated with the blue arrows in Fig. 3A. These latter features with positive slopes became more resolved in an applied magnetic field, seen at $B = 5$ T (Fig. 3B) and 8 T (Fig. 3, C and D), resembling the spectral signatures of QDs (12, 19). [See supplemental data (18) for a comparison of the gate maps on supported and suspended graphene].

To further explore the analogy with QD physics, we examine the energy scales in the spectral maps of the suspended graphene. As an example,

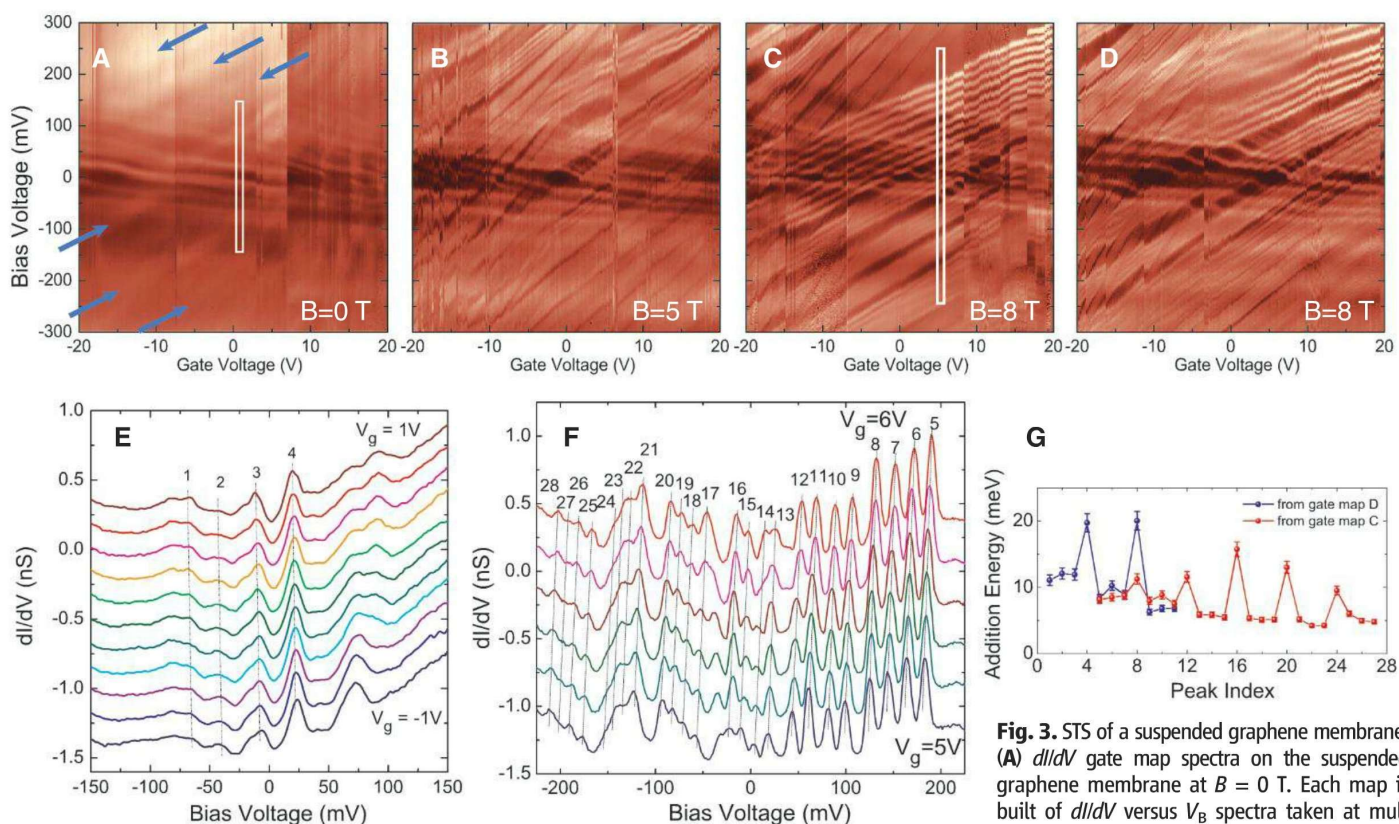


Fig. 3. STS of a suspended graphene membrane.

(A) dI/dV gate map spectra on the suspended graphene membrane at $B = 0$ T. Each map is built of dI/dV versus V_B spectra taken at multiple fixed gate voltages. The color scale is the

dI/dV magnitude, which varies from 0.05 nS (dark) to 1.7 nS (bright) for (A) and to 1.2 nS (bright) for (B) to (D). The blue arrows indicate spectral bands with positive slope that become more resolved at higher fields in (B) and (C). (B and C) dI/dV gate-map spectra on the suspended graphene membrane at (B) $B = 5$ T and (C) $B = 8$ T. The white rectangle in (C) denotes the region where individual spectra are obtained and plotted in (F). (D) dI/dV gate-map spectra at $B = 8$ T, showing the variability in the measurements when moving off and back on the membrane. (E) dI/dV versus V_B spectra from the $B = 0$ T gate map in (A) for gate voltages varying from -1 to 1 V. (F) dI/dV versus V_B spectra from the $B = 8$ T gate map in (C) for gate voltages varying from 5 to 6 V. The spectra are shifted vertically for clarity in (E) and (F). (G) QD addition energies corresponding to the difference in dI/dV peak positions in the spectra from the gate maps in (C) (red symbols) and (D) (blue symbols). Energies are converted from bias voltages using the lever arm, $E = \alpha V_B$, where $\alpha = 0.45 \pm 0.03$. The error bars in (G) are dominated by the statistical error in α , which was determined from 1-SD uncertainties in the measured slopes of the charging lines in the gate maps. These uncertainties, in turn, determine the corresponding uncertainties in the capacitance ratios discussed in the main text.

dI/dV spectra that are part of the gate maps in Fig. 3, A and C, are shown in Fig. 3, E and F, respectively. The peaks in Fig. 3E corresponding to the horizontal lines in the zero-field gate map (Fig. 3A) showed a separation of ≈ 30 mV for the first few states. In contrast, the peaks in Fig. 3F that form the positive-slope bands were seen in groups of four with a spacing of ≈ 20 mV. This grouping is similar to the fourfold charging peaks in QDs observed in graphene (12) or carbon nanotubes (19) and reflects the fourfold degeneracy of the dot levels caused by electron spin and valley quantum numbers. These dI/dV peaks are caused by an opening up of a transport channel at the Fermi energy associated with a single-electron addition to the QD. The peaks appeared as tilted lines in the map because the QD energy levels are controlled by a linear combination of the gate voltage and tip bias.

Here, we use this analogy to determine the energy scale and the size of the QD from the measured slopes and spacing of the charging lines. The vertical voltage bias axis is converted to the QD energy spacing using the lever arm, $E = \alpha V_B$, where $\alpha = C_B/C_T \approx 0.4$, C_B is the graphene layer to graphene QD capacitance, and

C_T is the total capacitance of the QD. In Fig. 3G, we plot the energy difference between the levels from the gate maps in Fig. 3, C and D (red and blue symbols, respectively). The energy spacing of the individual levels follows the classic energy spectrum of a QD (20), $e^2/C_T + \Delta E_{N+1}$, where e is the charge on an electron, e^2/C_T is the charging energy of the fourfold degenerate levels of a graphene QD (base line in Fig. 3G), and the additional energy, $\Delta E_{N+1} = \Delta E_{N+1} - \Delta E_N$, separating each band (spikes in Fig. 3G) corresponds to the energy needed to reach the next QD level (where N is the current quantum level). The charging energies, varying from 11.7 ± 0.5 to 5.3 ± 0.7 meV in Fig. 3G, correspond to QDs with diameters in a range from 34 ± 2 to 53 ± 5 nm (21). This dot size is consistent with a simple estimate based on the number of electrons added to the dot for a given range of gate voltage. In the gate map (Fig. 3C), we see approximately five quartet bands over the gate-voltage range of 40 V, corresponding to the addition of 20 electrons to the QD. The gate capacitance of 5.85 nF cm^{-2} (100-nm vacuum plus 200-nm oxide) yields a rough estimate of the dot diameter of 42 nm.

The profile of the membrane as measured by the tip height cannot explain the formation of a QD. To understand the local and global membrane deformation and the corresponding strain induced in the immediate vicinity of the STM tip, we performed molecular dynamics simulations (18). The calculations involved approaching the membrane with the tip at three different horizontal locations along a radial line across the membrane (2.5 and 5.2 nm from the edge, as well as in the center of the membrane) and then retracting the tip until a critical point was reached, at which further retraction of the tip would result in loss of tip-membrane contact (18). This critical point simulates the action of the STM servo that redraws the tip from the membrane to avoid contact while it maintains a vacuum tunneling gap. Additionally, a constant force of magnitude in the range 0 to 0.06 pN was applied to each carbon atom of the membrane to simulate the electrostatic force exerted by the gate electrode. The range of the back-gate force was selected such that different levels of membrane deformation took place above and below the neutral position (fig. S9) (18).

Figure 2E illustrates the atomistic model that we used, showing the tip at the critical height above the pulled-up membrane. In Fig. 2F, the membrane shapes at the critical tip height are plotted together for three tip locations on a membrane, for a back-gate force of 0.012 pN per atom. The computational simulations show that the deformation in the graphene membrane induced by the probe tip is formed locally. The simulations further demonstrate that the measured membrane profiles are not caused by a static membrane deformation, but one that continuously changes with the tip position. As the STM tip is rastered across a membrane, the cusp of the deformation follows the tip (Fig. 2F), and the

domelike shapes recorded in STM images (Fig. 2, A to D) are the envelopes tracing the cusp peak, as illustrated by the dot-dashed line in Fig. 2F.

The mechanical simulations also show that the deformation in the graphene lattice induced a strain in the membrane (Fig. 4A), which was localized on the small scale of the probe-tip diameter (5 nm) used in the simulation. A scaling analysis (figs. S12 and S13) (18) shows the deformation area scales with the square root of the probe-tip diameter for a flat membrane. Extrapolating the deformation size for the experimental probe diameter of 100 nm (determined by scanning electron microscopy) yields a strain field diameter of ≈ 23 nm for the current experiment. Because the local deformation of the membrane under the tip does not change substantially as the probe tip moved across the membrane (Fig. 2F), the local strain field is fairly constant at all tip positions. The local deformation is also expected to be fairly independent of gate voltage, because the cusp of the deformation is controlled largely by the van der Waals force from the probe tip (fig. S9) (18).

Peak areal strains up to $\approx 1\%$ are predicted (Fig. 4A) in the graphene lattice directly under the tip. This strain produces pseudofields that could directly affect the graphene charge carriers (3–11). The symmetry of the pseudofields is determined by the corresponding symmetry of the strain field. For example, a uniform pseudomagnetic field requires a special strain field (4) distorted with threefold symmetry. In our case, a rotationally symmetric strain field generates a threefold pseudomagnetic field with alternating signs (6, 7). Figure 4B shows the calculated pseudomagnetic field for the strain field in Fig. 4A for the suspended membrane resulting in alternating spatial fields of ± 10 T. The region where the pseudofield is maximal is ≈ 10 nm in diameter for the 50-nm membrane diameter used in the simulation. The use of the same scaling as described above results in an effective pseudofield diameter of ≈ 45 nm (18).

Carriers in graphene can penetrate large potential barriers due to the effect of Klein tunneling (22), and physical barriers are typically engineered (23) to confine carriers. The pseudomagnetic field spatially confines the graphene carriers curving the classical trajectories and forming clockwise and counterclockwise orbits around the alternating peaks of the pseudomagnetic field (6, 7). However, some electronic states corresponding to classic snake orbits that propagate along the lines where the pseudomagnetic field changes sign will not be confined. We suggest that the application of an external magnetic field suppresses such leaky orbits by canceling one component of the pseudomagnetic field that opposes the applied magnetic field, which improves the overall confinement, as observed in Fig. 3, B to D. The dot size estimated from the charging energies in the gate maps (Fig. 3G) is ≈ 34 to 53 nm in diameter, which is in reasonable

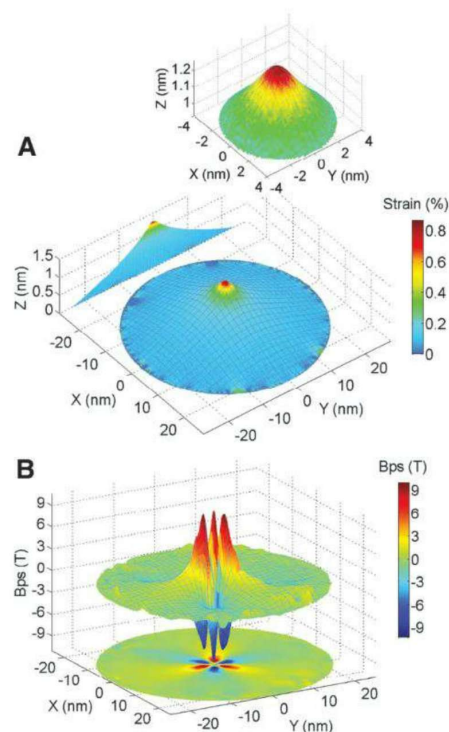


Fig. 4. Simulations of a graphene membrane shape and corresponding strain and pseudomagnetic field. **(A)** Graphene membrane shape with the STM tip positioned over the center of the membrane at zero back gate force. The inset shows a zoomed-in region where the strain is maximal. The radii of the tip and membrane in this model are 2.5 and 25 nm, respectively. **(B)** The pseudomagnetic field, calculated from the strain in (A) (fig. S10) (18), shows a spatially alternating field with threefold symmetry that can spatially confine carriers.

agreement with the size of the estimated pseudofield (45 nm). The density-of-states peaks observed as weakly negative sloping lines in the gate maps (Fig. 3A) may be caused by localization in a spatially varying pseudofield (6, 7, 10), although a detailed model is currently not available. The variations in positive-slope bands (Fig. 3, B to D) are probably caused by minor deformations of the overall membrane shape affecting the size of the QD.

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Acknowledgments: We thank M. Stiles and S. Adam for valuable discussions and S. Blankenship and A. Band for technical assistance. The U.S. National Science Foundation is gratefully acknowledged via grants CMMI-1069076 and CMMI-1129826 (T.L. and S.Z.) and grant CMMI-0841840 (C.A.W. and S.D.S.).

Supplementary Materials

www.sciencemag.org/cgi/content/full/336/6088/1557/DC1
Supplementary Text
Figs. S1 to S13
References (24–26)

9 February 2012; accepted 30 April 2012
10.1126/science.1220335

Electrical Wind Force–Driven and Dislocation-Templated Amorphization in Phase-Change Nanowires

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Phase-change materials undergo rapid and reversible crystalline-to-amorphous structural transformation and are being used for nonvolatile memory devices. However, the transformation mechanism remains poorly understood. We have studied the effect of electrical pulses on the crystalline-to-amorphous phase change in a single-crystalline Ge₂Sb₂Te₅ (GST) nanowire memory device by in situ transmission electron microscopy. We show that electrical pulses produce dislocations in crystalline GST, which become mobile and glide in the direction of hole-carrier motion. The continuous increase in the density of dislocations moving unidirectionally in the material leads to dislocation jamming, which eventually induces the crystalline-to-amorphous phase change with a sharp interface spanning the entire nanowire cross section. The dislocation-templated amorphization explains the large on/off resistance ratio of the device.

Chalcogenide-based phase-change memory (PCM) materials have been widely used for optical data storage and are now finding applications in electronic memory devices (1, 2). In a nonvolatile PCM device, the difference in optical reflectivity or electrical resistance between amorphous and crystalline phases is used to store information. Memory switching is performed by applying optical (or electrical) pulses: short pulses with large amplitude to amorphize the material and long pulses

with low amplitude for the crystallization process. It has been generally assumed that the large amplitude of the pulse melts the material, and its short duration locks the atoms in their disordered positions owing to rapid quenching. The understanding of the effects of electric field on the crystalline-to-amorphous phase transition in PCM is critical for designing low-power nonvolatile memory devices. Therefore, it is desirable to visualize and characterize the critical events that lead to the phase-change process while the device is under operation, which can uncover phenomena that cannot be gleaned from ex situ measurements (3). However, it is challenging to resolve the amorphization phenomena with high spatial and/or temporal resolution in confined structures such as in conventional thin-film devices with a sandwich geometry (4). Very recently, based on time-resolved photo-excitation measurements of PCM materials (5, 6), it has been argued that the material did not amorphize

via the liquid-state pathway, but by a solid-state, lattice-distortion-triggered process. Consequently, visualization of the structural evolution of the PCM device under electrical biasing by correlating it to the electrical-resistance variation would be helpful. A single-crystalline device can serve as an ideal platform to study the relationship between microstructural evolution and electrical resistance variation, so as to avoid the effects of preexisting grain boundaries on carrier transport and structural dynamics. In conventional PCM devices, polycrystalline thin films with relatively small grain sizes (10 to 20 nm) preclude the visualization of switching behavior originating from single grains. By using the single-crystalline, lateral, and open geometry of the Ge₂Sb₂Te₅ (GST) nanowire structure, we visualized the amorphization behavior and found it to be critically associated with the nucleation and dynamics of dislocations.

We assembled GST nanowire memory devices across 2-μm-wide trenches fabricated on a thin silicon-nitride membrane (Fig. 1A) to enable real-time visualization of nanoscale structural changes with high spatial resolution under the influence of applied electrical voltage pulses inside a transmission electron microscope (TEM) (figs. S1 to S4) (7). Single-crystalline GST nanowires with hexagonal crystal structure grown along the [10 $\bar{1}$ 0] axis were synthesized through the vapor-liquid-solid method (fig. S1) (8). For device operation, voltage pulses with duration of a few hundred nanoseconds were applied, and between each pulse, the resistance was measured at a DC bias of 0.2 V (Fig. 1B). We note that all the reported electrical resistances in this paper are not the dynamic resistances during electrical pulsing, but the stationary resistance values of the devices measured long after the pulsing process so that the heat generated during the pulsing was dissipated and the devices reached room temperature. The resistance (after each 300-ns pulse with increasing voltage amplitude)

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of a 280-nm-thick GST nanowire device as a function of the pulse amplitude (Fig. 1C, blue squares) decreases slightly above 3.3 V, just before amorphization electrical switching was observed. The resistance dip, however, was very small as compared with the more than two orders of magnitude increase in the electrical resistance associated with amorphization switching. There are some critical observations related to the resistance dip: (i) The decrease in the electrical resistance reflects a permanent structural change in the device while still in the crystalline state; and (ii) the critical voltage at which the resistance starts to decrease is a function of the nanowire thickness and pulse width (Fig. 1C and fig. S5). The voltage at which the resistance dip initiates decreased with increasing pulse width, and thicker wires required higher voltages and longer pulses (Fig. 1C, inset).

To correlate the onset of the resistance dip and amorphization with structural evolution, we show snapshots of the dark-field (DF) TEM movie (movie S1) and the corresponding electrical resistances of a nanowire device in Fig. 2. The resistance dip before amorphization was a general feature of all nanowire devices measured either inside or outside the TEM. For a 230-nm

nanowire device, no structural changes in the DF TEM images were identified until ~ 5 V pulses (800 ns), but concomitant with the onset of the resistance dip, the uniform contrast of the single-crystalline device started to change dynamically, with extended line-shaped defects (Fig. 2B) and other strain-related contrasts. For voltages below the resistance dip (<5.8 V), the contrast continued to change upon each applied pulse, but no directional motion was observed. High-resolution TEM characterization of the line defects reveals the presence of dislocations (fig. S6) with a Burgers vector of $\frac{1}{2}\langle 11\bar{2}0 \rangle$. Considering the hexagonal-crystalline structure of GST grown along the $[10\bar{1}0]$ axis, the line-shape structure represents the projection image of the dislocation loop that lies on the $(10\bar{1}0)$ prismatic plane, as illustrated in Fig. 1A.

Notably, upon further increasing the voltage pulse amplitude (800 ns duration), the resistance dip was directly associated with the line defects becoming mobile and propagating along the direction of hole-carrier motion (Fig. 2, C to F). This observation suggests the influence of the carrier wind force to break the symmetry of the nanowire for dislocation motion, thereby inducing a polarity-dependent behavior (9–11). The

resistance continued to drop with the increase in the pulse amplitude, accompanied with the motion of dislocations from the positive to the negative electrode. In particular, dynamic contrast changes were observed moving unidirectionally in the device. Upon approaching the bottom of the resistance dip, it was observed that the dislocations stopped moving (jamming), followed by the formation of highly accumulated and entangled dislocations at the jammed location (Fig. 2, G and H, and movie S1). Finally, amorphization switching was observed, accompanied by an increase in the electrical resistance by approximately two orders of magnitude. A clear bright line was identified as the amorphous phase (red arrow, Fig. 2I), which was further characterized by electron diffraction studies (fig. S7). TEM image of the free-standing region of the nanowire (over the trench) in Fig. 2J shows a trail of dislocation cloud right behind the amorphized region. In addition, a few other dislocation cloud trails were identified along the nanowire but without any amorphous marks, which indicates that they got jammed by random perturbation in the nanowire structure, with one among them eventually undergoing amorphization. These observations show that the entangled dislocations

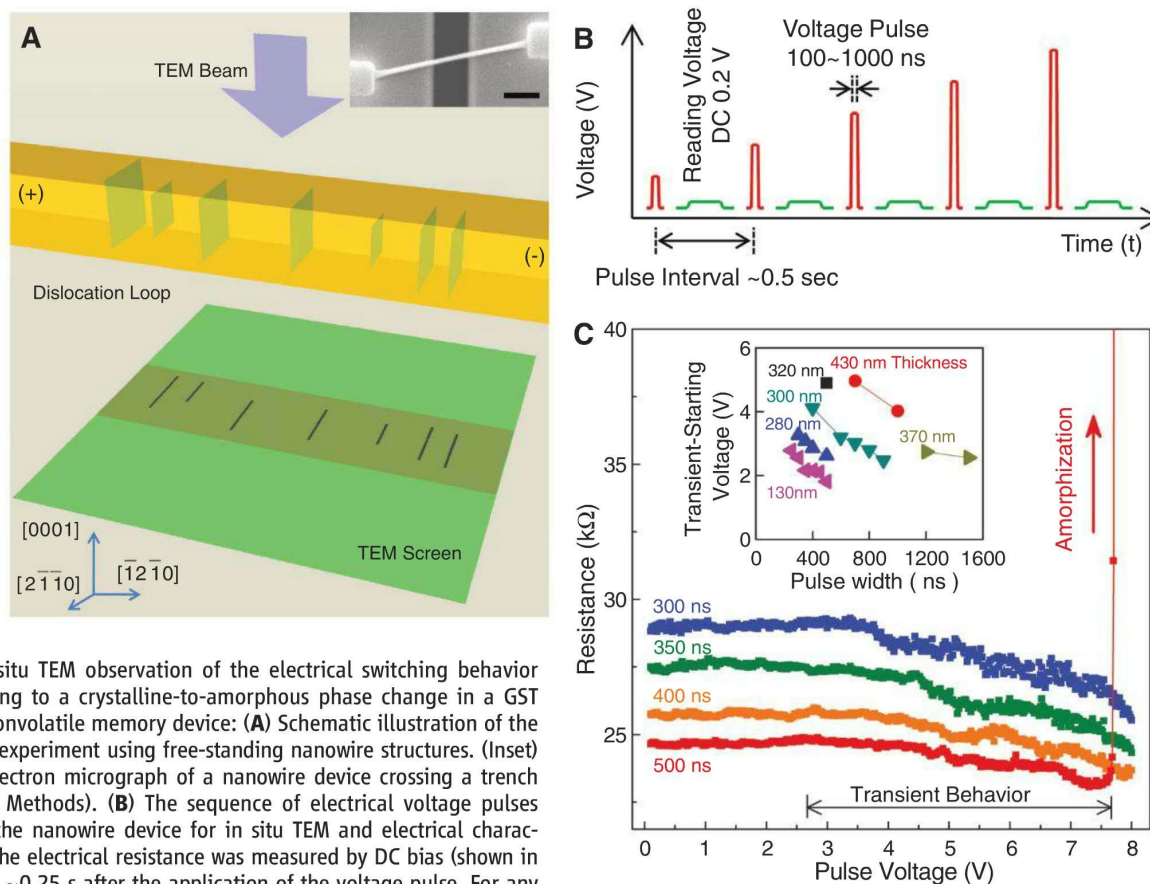


Fig. 1. In situ TEM observation of the electrical switching behavior corresponding to a crystalline-to-amorphous phase change in a GST nanowire nonvolatile memory device: (A) Schematic illustration of the in situ TEM experiment using free-standing nanowire structures. (Inset) Scanning electron micrograph of a nanowire device crossing a trench region (see Methods). (B) The sequence of electrical voltage pulses applied to the nanowire device for in situ TEM and electrical characterization. The electrical resistance was measured by DC bias (shown in green) after ~ 0.25 s after the application of the voltage pulse. For any pulse sequence, the amplitude of the applied voltage pulse was increased with a 0.5-s interval between any two pulses while their widths were kept constant. (C) Plots of electrical resistance of the nanowire device versus applied voltage pulse amplitude obtained for different pulse width sequences, starting from a single-crystalline state. Resistance dip was observed before amorphization switching. (Inset) A plot of nanowire size versus the voltage at which the resistance dip is initiated as a function of applied pulse widths.

quenes, starting from a single-crystalline state. Resistance dip was observed before amorphization switching. (Inset) A plot of nanowire size versus the voltage at which the resistance dip is initiated as a function of applied pulse widths.

caused by dislocation generation and jamming are precursors for the amorphization switching of the device.

To confirm that the electrical polarity determines the direction of propagation of dislocations by the carrier wind force, we performed a “reverse-bias” experiment with the opposite polarity applied on the same device (Fig. 2, K to T, and movie S2). The device was first switched back to the crystalline state, followed by the continuous application of electrical pulses with the opposite polarity in the same sequence as described above. Upon applying 500-ns-width pulses, the resistance dip was observed, during which the jammed dislocation cloud from the

previous measurement was relieved by moving in the opposite direction. We then stopped pulsing at 5 V, and then applied 800-ns pulses starting from 0 V. The device resistance started from the resistance at which we stopped the 500-ns pulses (Fig. 2K), which then began to dip again from ~2 V (longer pulses lead to onset of resistance dip at lower voltages). Dynamic contrast changes were observed to move in the opposite direction, which again corresponds to the direction of the hole carriers (movie S2). Eventually, amorphization switching occurred at ~5.2 V (Fig. 2, P to S), with the dislocation cloud appearing behind the amorphous mark, consistent with the forward-bias experiments (Fig. 2T). The reverse-bias

pulsing experiment clearly indicates that the mobile dislocations are transported in the direction of the applied bias, which is the direction of the motion of hole carriers in the device, suggesting a critical role of dislocation nucleation, motion, and jamming in creating the amorphous phase in the PCM device.

To better visualize the amorphization switching process, we sculpted a notch structure in the nanowire suspended over the trench to localize the phase-change region at the notch (Fig. 3) (3). In bright-field (BF) TEM image, the as-fabricated nanowire appeared uniformly dark owing to its single crystallinity (Fig. 3A). During electrical pulsing (movie S3), a change in the

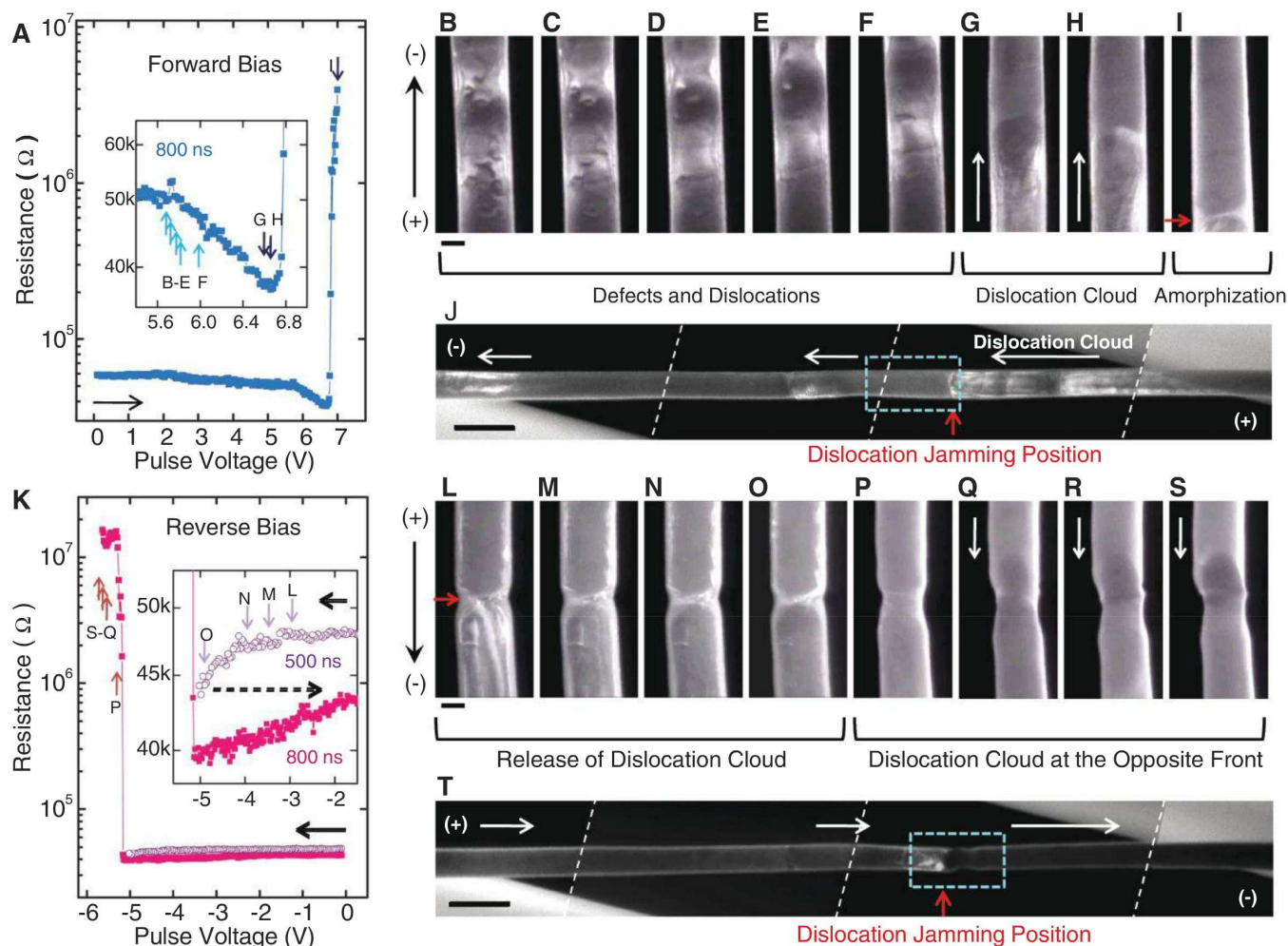


Fig. 2. Electric wind force–driven mobile dislocations and amorphization switching via in situ TEM measurements: (A to J) “Forward-bias”; (K to T) “reverse-bias,” i.e., opposite polarity from the same region of the device. (A) Plot of stationary electrical resistance of the nanowire device versus voltage pulse amplitude for the forward-bias direction. The electrical resistance states are correlated with the TEM images of the device, as indicated by arrows. (B to I) Snapshots of DF TEM images (from movie S1) during electrical switching: At the early stage, individual dislocations were identified in (B) to (E), which started to propagate along the hole-carrier motion direction (F to I). The white arrows indicate the unidirectional motion of the TEM image contrast. At the bottom of the electrical resistance dip, a dislocation cloud was observed in (G) and (H). Following the resistance dip regime, amorphization occurred at the dislocation

jamming region [red arrow in (I)]. (J) Larger-area DF TEM image of the free-standing GST nanowire device after amorphization, showing a dislocation trail behind the amorphized region. The blue rectangular area was recorded in the TEM movie (movie S1). (K) Plot of electrical resistance versus voltage pulse amplitude for the reverse-bias case. (L to S) From movie S2; the dislocation cloud behind the jamming region was first relieved (L to O), and subsequently another dislocation cloud was developed on the opposite front of the jamming transition region (P to S). (T) Larger-area DF TEM image of the nanowire after the reverse-polarity–biased amorphization switching, again showing a dislocation trail behind the amorphized region, but in the opposite direction as compared with (J). Scale bar: (B to I) and (L to S), 100 nm; (J) and (T), 500 nm. The dashed lines in (J) and (T) denote the regions where the images were combined.

image contrast was observed while the device was still in the crystalline state, with the notch region developing a dark line contrast (Fig. 3B), followed by a contrast variation moving from the positive- to the negative-electrode side (Fig. 3C). The image contrast variation along the nanowire was again correlated with the resistance dip, consistent with our previous observations for the non-notched devices. Finally, upon amorphization switching, the dark line in the middle of the notch was abruptly changed into a bright amorphous band (Fig. 3D). Microstructure analysis (BF TEM images) both before and after the switching (fig. S8) shows that a highly strained structure developed in the notch region before amorphization. In particular, the amorphous mark appeared in this region of strain, which suggests the role of strain in the amorphization process. The origin of the strained structure and the differential contrast was further studied by DF TEM analysis (Fig. 3E). DF TEM revealed the presence of a high density of dislocations, predominantly at the positive-electrode side of the notch (Fig. 3E). Even though the notch structure was intended to localize joule heating by enhancing the current density, our in situ observation additionally suggests that the notch was also more likely to behave as a geometrical necking region where mobile dislocations were jammed and accumulated by the electrical wind force that did not pass through the constriction easily (see also fig. S9 for results from another device).

In contrast to thin-film polycrystalline GST, which consists of small grains (10 to 20 nm), the single-crystalline nanowire structure rules out the effect of preexisting grain boundaries for both structural evolution and resistance dip. Our observations show that the dislocations are continuously nucleated along the single-crystalline GST nanowire, which become mobile above a certain applied voltage that is correlated to the resistance dip. The voltage pulses play a critical role by inducing heat shocks along the single-crystalline nanowire, which create dislocations by vacancy condensation (12). The rising edge of the pulse produces lattice vacancies in the material caused by heating, with the nanowire surface/interface serving as the lattice vacancy source, whereas during rapid cooling, atomic vacancies are condensed into dislocation loops by nonconservative dislocation climb (12, 13) (fig. S10). This mass-action dislocation generation path is different from purely displacive shear nucleation driven by an external mechanical stress (14–18). Because our nanowire is a free-standing structure where the thermal-expansion stress is relieved from its surface (19) and by buckling, the dislocation formation is attributed more to vacancy supersaturation (osmotic forces) than to mechanical stress. After formation, these dislocations feel the directional electrical wind force, but display a threshold behavior in mobility: that is, as a whole, they only start to move when the electrical wind force exceeds a critical

value. When the dislocations become mobile, they consume more vacancies along the way by combined climb and glide actions, which decrease the carrier scattering probability, thereby decreasing the electrical resistance of the device (Fig. 1C) (13). (Dislocation dynamics are illustrated in figures S11 to S14.) Similar electrical resistance drops have been observed during rapid annealing of metals, attributed to Frank and prismatic dislocation loop nucleation and growth driven by vacancy condensation (13). Therefore, we believe that an electrical current pulse has two physical effects, one directional and the other nondirectional. The nondirectional thermal heating and quenching produce the dislocations, whereas the directional wind force moves the dislocations in the drift direction of the majority charge carrier (holes in the case of GST), leading to polarity dependence. Yang *et al.* (10) have reported that hole-carrier wind force in GST

induces a mass transport by electromigration at high current densities, which partly corroborates our observation of polarity-dependent dislocation motion.

One question that arises naturally is, what specific properties of GST lead to the observation of dislocation nucleation and transport, which is quite different from those in other covalently bonded materials such as silicon? To understand dislocation transport in crystalline hexagonal GST, we performed *ab initio* density functional theory (DFT) calculations to characterize slip systems of a prismatic dislocation loop. The DFT calculations for basal (0001)[1120] and prismatic (1100)[1120] slips exhibit unstable generalized-stacking-fault (GSF, γ) energies of 10.1 and 172.8 mJ/m², respectively (Fig. 4A and fig. S15). $\gamma_{\text{unstable}}^{(0001)[1120]}$ is extremely small [similar to basal slip in graphite (20)]; in GST-layered structures with

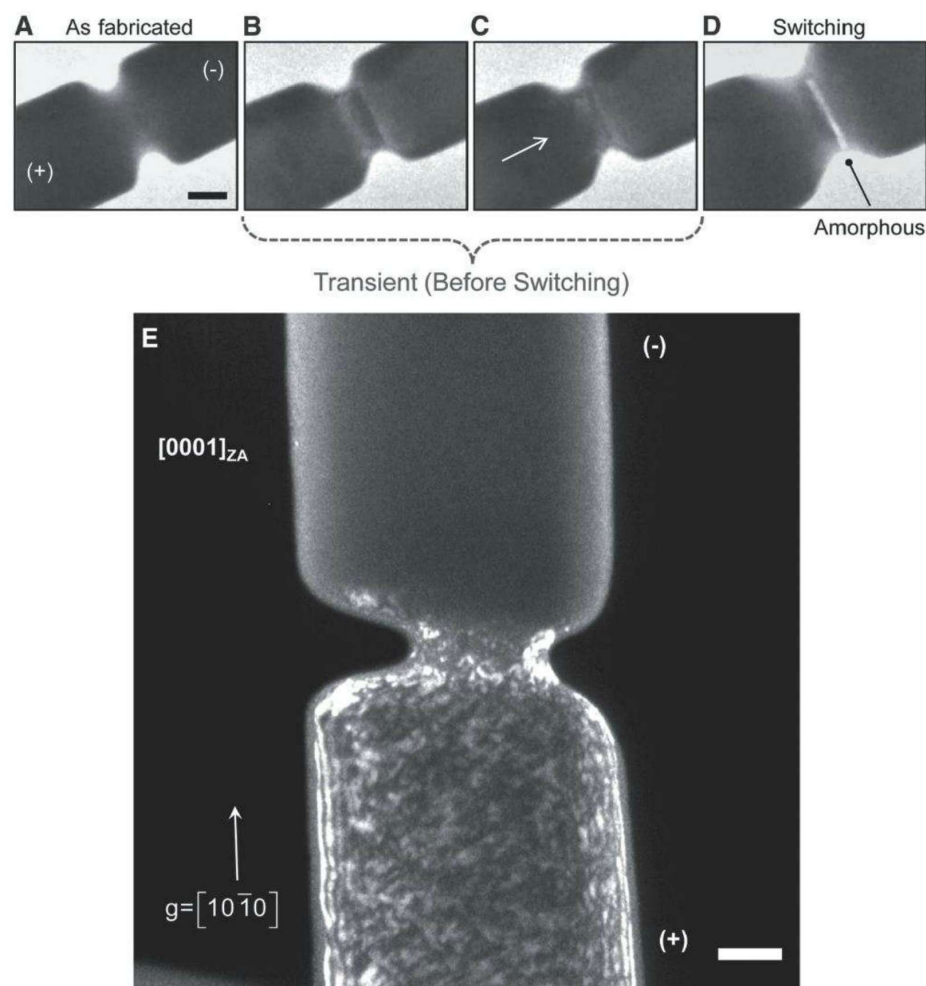


Fig. 3. Transient behavior (region of resistance dip) followed by amorphization switching for a nanowire device with a notch (constriction). (A to D) Snapshots from BF TEM movie (movie S3): As-fabricated nanowire device shows a uniform, dark contrast along the nanowire in (A). As the electrical pulses were applied, the variation in TEM image contrast was identified at the moment of the initiation of the resistance dip. Darker line in the middle of the notch in (B) and (C) shows a region of highly concentrated strain, which was changed into bright contrast upon amorphization (D). (E) DF TEM image of the single-crystalline nanowire close to the bottom of the transient resistance dip: A dislocation cloud was located mostly at the (+) electrode region in (E). Scale bars (A and E), 100 nm.

a stacking sequence -Sb-Te-Ge-Te-Te-Ge-Te-Sb-Te (also occurs in cubic GST) (21), weak Te-Te bonding on the basal plane is the most likely to slip because of its long bond length and weak bonding energy (22). $\gamma_{unstable}^{(1100)[1120]}$ is quite small also, comparable to that for partial dislocation slip in Cu (169 mJ/m^2) (23), and much smaller than those for slips in Si ($>1500 \text{ mJ/m}^2$) (24); thus, motion of prismatic dislocation loops driven by the electrical wind force is feasible in GST (for crystallographic details on glide of prismatic loops, refer to figs. S11 to S14).

We summarize the dislocation generation and dynamics leading to amorphization switching in Fig. 4B. Both heat (nondirectional) and carrier wind force (directional) effects are consequences of the applied voltage pulses. Just before the initiation of the resistance dip, the heat-shock process by the electrical pulses leads to generation of vacancies followed by their rapid condensation to generate dislocations, analogous to generation of Frank-partial and prismatic dislocations in face-centered cubic metals by vacancy condensation (25). From our observation, it is likely that most of the dislocations are generated at the positive-bias metal electrode in contact with the nanowire (owing to material incompatibility and larger potential drop), but some of them can also be generated along the nanowire. As the amplitude of voltage pulses increases, the carrier wind force makes the dislocations mobile, gliding as prismatic dislocation loops.

These dislocation loops grow in size as they move, encounter, and annihilate more vacancies, acting as “garbage collectors,” which decrease the total disorder in the crystal, but also transport some disorder (in the form of dislocation cores) downstream in a one-dimensional (1D) traffic flow along the nanowire.

One-dimensional traffic flows are inherently fragile and prone to instabilities, because of interactions between the mobile agents that lead to density-dependent mobility (26). For instance, analytical models of automobile traffic on a highway predict sharp, catastrophic jamming when the automobile density exceeds a certain fraction of the maximum packing density (27). Owing to the mutual tangling that lowers their 1D mobility, the mobile dislocations moving in the direction of the hole drift can jam at some location. The dislocations then accumulate at the jammed region of the nanowire, which creates a high degree of disorder locally in the crystalline material, leading eventually to amorphization switching (28–31). Therefore, paradoxically, the mobile extended defects, which decrease the crystalline disorder elsewhere, can also greatly concentrate the disorder in a local region, when the jamming transition occurs. For nanowires without a notch, the dislocation jamming can occur anywhere when a critical dislocation density is reached in the system.

Our observations show that amorphization in PCM devices is templated by dislocation transport and jamming in the crystal. If the devices

were amorphized by conventional melt-quench process, then one would have expected initiation of melting from the nanowire surface that should have spread, eventually leading to an amorphous shell around a crystalline core. Such a mechanism would have led to poor on/off resistance ratios because of resistors in parallel. In contrast, we have always observed the amorphous phase form in series with the crystalline phase (3), with the amorphous volume occupying just a tiny fraction of the total device volume, yet achieving good device performance with large on/off resistance ratios. Furthermore, in all the measured devices, the amorphous mark always appeared in front of the jammed dislocation cloud spanning the entire cross section of the nanowire and with very sharp vertical interfaces, with no amorphous phase seen anywhere else. It is thus evident that the dislocation jam with vertical boundaries provides a microstructural template for amorphization to occur in a vertical section, which is different from conventional understanding of melting that is templated by free surfaces. Solid-state amorphization is known to occur in silicon (29) and metallic (31) crystals by mechanical ball milling that injects a high density of dislocations into the material, where melting is not possible at room temperature. We cannot completely rule out the possibility of transient melting in the dislocated region, because dislocations do increase the local resistance and heat generation. The physics of such atomic disordering around dislocation cores—i.e., premelting (32)—even if it occurs, would be quite different from normal melting, because the degree of atomic disordering around dislocation cores can only rise gradually with temperature, unlike a sharp first-order thermodynamic transition in a conventional bulk melting process. In any case, our main result, which is dislocation-templated amorphization of narrow cross section with a sharp interface, is microstructural—independent of the detailed chemical physics of atomic disordering—and consistent with the observed device performance (large on/off ratio). Our results, based on direct observation by in situ TEM analysis, are consistent with several recent reports (5, 6, 33) that also have suggested non- (conventional) melting-based amorphization process in GST material.

The observation of the voltage pulse-induced dislocation nucleation, wind force-assisted dislocation glide and jamming, and subsequent dislocation-templated amorphization shed light on previously unexplored properties of these fascinating materials, relating microstructural evolution to device properties. A similar mechanism could operate in polycrystalline thin-film devices as the grain boundaries can be considered as networks of dislocations and also act as dislocation sources and sinks. Furthermore, direct visualization of various processes occurring in PCM devices under electrical bias would be promising to understand their unusual fast switching behavior and will also be applicable to other functional materials and devices.

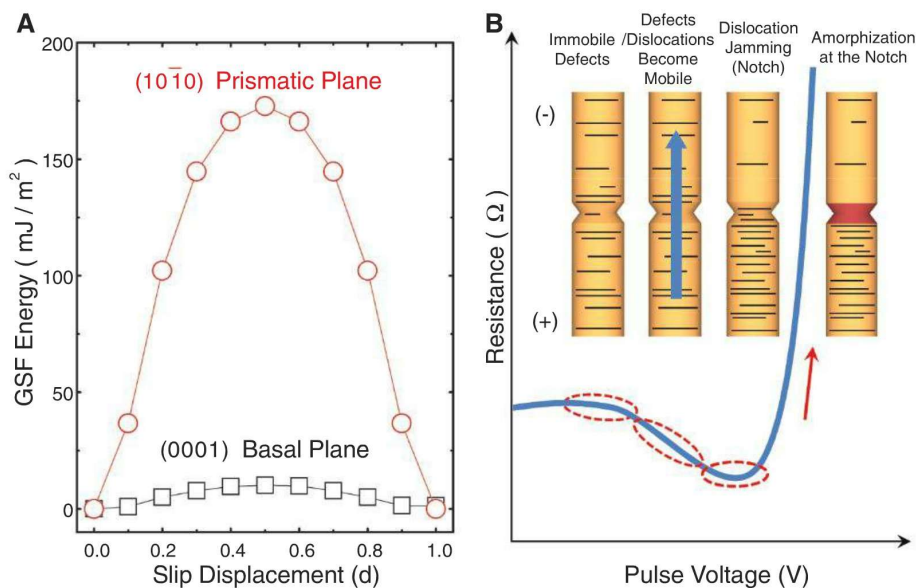


Fig. 4. (A) DFT calculation of generalized stacking fault (GSF) energy for both basal (0001) and prismatic (10 $\bar{1}0$) planes in hexagonal $\text{Ge}_2\text{Sb}_2\text{Te}_5$. (B) Our model of dislocation nucleation, transport, and jamming by electrical pulses: At the initiation of the dip of electrical resistance, the electrical pulses induce heat shocks to nucleate dislocations from atomic vacancies. As the pulse voltage amplitude increases, the dislocations become mobile and move along the direction of holes owing to the carrier wind force. Eventually, because of the increase in dislocation density, there is a jamming transition (e.g., in the notch region or elsewhere for regular wires), where the dislocations pile up, followed by amorphous switching in the same region. On the other side of the notch, the dislocations move away from the observed region in TEM owing to wind force.

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Acknowledgments: This work was supported by the Office of Naval Research (grant N000140910116), Materials Structures and Devices Center at the Massachusetts Institute of Technology, NSF (DMR-0706381 and DMR-1002164), and Penn-Materials Research Science and Engineering Center (DMR05-20020 and DMR11-20901). Y.C.L., L.Q., and J.L. acknowledge the support of NSF DMR-1008104 and DMR-1120901, and Air Force Office of Scientific Research FA9550-08-1-0325. Y.L. and A.T.C.J. acknowledge the support of the Nano/Bio Interface Center through NSF Nanoscale Science and Engineering Center DMR08-32802. Electron microscopy experiments were performed at the Penn Regional Nanotechnology Facility at the University of Pennsylvania. S.-W.N. thanks D. Strachan for useful discussions on in situ TEM experiments. The data described in the paper are archived by the Agarwal group at the University of Pennsylvania.

Supplementary Materials

www.sciencemag.org/cgi/content/full/336/6088/1561/DC1

Materials and Methods

Figs. S1 to S15

References

Movies S1 to S3

6 February 2012; accepted 18 May 2012

10.1126/science.1220119

Breaking the Speed Limits of Phase-Change Memory

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Phase-change random-access memory (PCRAM) is one of the leading candidates for next-generation data-storage devices, but the trade-off between crystallization (writing) speed and amorphous-phase stability (data retention) presents a key challenge. We control the crystallization kinetics of a phase-change material by applying a constant low voltage via prestructural ordering (incubation) effects. A crystallization speed of 500 picoseconds was achieved, as well as high-speed reversible switching using 500-picosecond pulses. Ab initio molecular dynamics simulations reveal the phase-change kinetics in PCRAM devices and the structural origin of the incubation-assisted increase in crystallization speed. This paves the way for achieving a broadly applicable memory device, capable of nonvolatile operations beyond gigahertz data-transfer rates.

Phase-change random-access memory (PCRAM) represents one of the best candidates for a so-called “universal memory” due to its nonvolatile nature, high scalability, low power consumption, high read/write speeds, and long read/write endurance (1–4). PCRAM operations, based on the reversible switching of phase-change (PC) materials between amorphous and crystalline states (5, 6), are generally fast: on the order of nanosecond time scales (7–9). However, the crystallization speed is much slower than the amorphization speed, which limits

its the overall writing speed of PCRAMs. Despite efforts (10–16) to increase the crystallization speed, it has been difficult to achieve a speed faster than 1 ns. A difficulty arises from the contradictory nature of increasing the crystallization speed while extending the stability of PC materials for long-term data retention (17). This has restricted the selection of PC materials in the commercial development of PCRAMs, typically emphasizing high stability at the expense of crystallization speeds.

According to classical nucleation theory, the nucleation of small crystallites and their subsequent growth are the two main distinct processes in crystallization (18–20). The nucleation rate is faster at lower temperatures, followed by rapid growth at higher temperatures (21). We show that by stimulating and altering these processes, we are able to control the speed of crystallization by electrical means. Our approach is based on the idea of providing a weak electric field to induce thermal prestructural ordering (incubation of ordered clusters in the amorphous matrix) via Joule heating, which enables faster nucleation and

growth upon a subsequent stronger electrical pulse, while maintaining the high stability of the amorphous phase by controlling the cluster-size distribution (Fig. 1). This thermal-incubation model is very different from the model of Karpov *et al.* (22), which assumes a direct electric-field-induced modification of the crystal-nucleation barriers in $\text{Ge}_2\text{Sb}_2\text{Te}_5$ (GST).

We experimentally fabricated porelike structured PCRAM cells (with GST as the PC material) and used these cells to study the incubation effects on the crystallization speeds (Fig. 1D) (23). To study the ultrafast switching effects, a weak electric field (equivalent to ~ 0.3 V), for tailoring the crystallization kinetics (hereafter referred to as the incubation field), was employed to achieve optimal switching properties without activating spontaneous crystallization (fig. S2). We employed subsequent electrical pulses (varying in length from several hundred picoseconds to several tens of nanoseconds) to switch the cells, and we used the full width at half maximum (FWHM) values of the pulses to characterize the switching speeds of the cells (fig. S3). The duration of a pulse experienced in the cell was confirmed to be the same as that measured before the pulse enters the cell (fig. S4).

We found that the incubation field, even with the small amount of thermal energy it delivers, can substantially promote the nucleation and growth of PC materials. Nucleation and growth times can be characterized by the minimum electrical pulse width needed to switch the cell from the amorphous state to the crystalline state (24), also known as the “set” process. When the incubation field is applied to a cell (Fig. 2A), much faster nucleation and growth are observed, as manifested by the substantial decrease in pulse width (by ~ 5 ns). For the fastest nucleation and growth, we found the shortest pulse to be 500 ps. This is approximately one order of magnitude

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faster than the fastest switching speeds previously achieved in GST/GeTe cells with similar sizes under full recrystallization conditions and with the use of the voltage-peak FWHM as a measure of the pulse duration (7, 8).

Fast crystallization speeds facilitated by an incubation field can be achieved without affecting the amorphization of PC materials (the

“reset” process). The reset process involves the melting of the material at high temperatures, followed by the rapid quenching of the material to the amorphous state. The reset process under an incubation field is also very fast. Amorphization speeds as fast as 500 ps were also achieved (fig. S5). More importantly, upon applying the incubation field, we discovered that a cell can be

switched reversibly and stably with set and reset pulses both equal to 500 ps for 10^4 cycles (Fig. 2B). We also observed that both the resistance of the amorphous state and the reset/set resistance ratio are relatively constant during the cycling experiment. These results show that both fast speed and stability of the amorphous phase can be achieved simultaneously.

Fig. 1. Prestructural ordering effects on the crystallization of PC materials. **(A)** Model configurations demonstrating the atomic rearrangements during the phase transition, with and without prestructural ordering. **(B)** Schematics of the crystallization probability as a function of temperature for PC materials. The nucleation and growth processes are accelerated when there is prestructural ordering (from method 1 to 2). **(C)** Waveform of a small-field incubation voltage and main pulse applied to set the PCRAM. A small voltage is first applied to initiate prestructural ordering, followed by a main pulse to induce crystal nucleation and growth of the PC material. **(D)** Schematic of the PCRAM structure with the pulse signal delivered to heat and crystallize the PC material (GST).

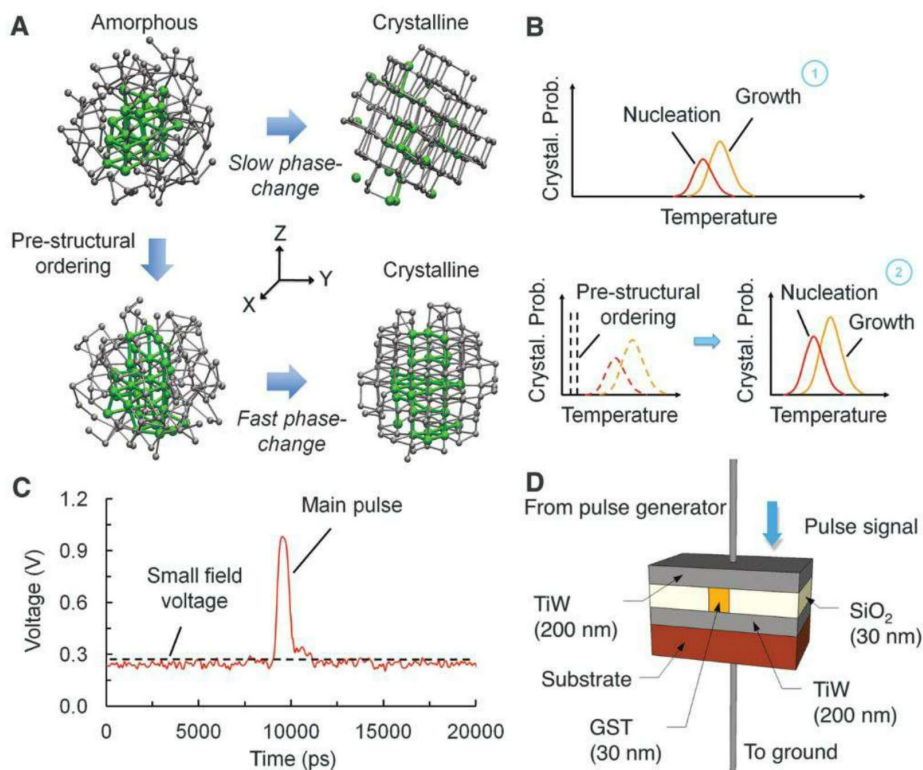
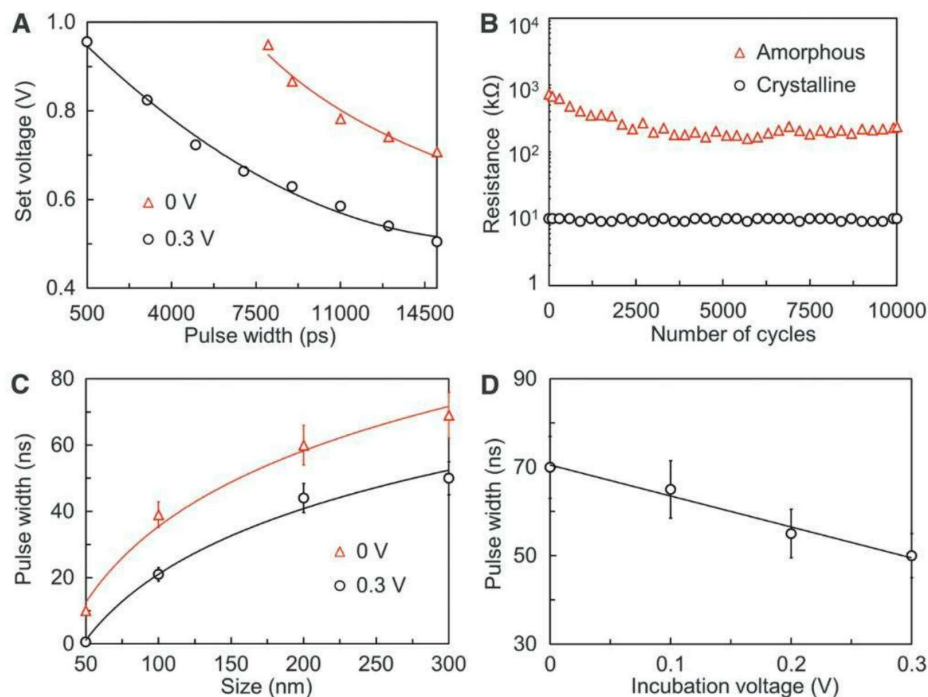


Fig. 2. PCRAM switching properties. **(A)** Dependence of minimum voltage on the pulse width achieved by a PCRAM cell (50 nm) under incubation-field conditions (0.3 V). The fastest speed achieved was 500 ps. **(B)** Reversible switching of PCRAM. Fast and stable switching with 500-ps pulses for both set (1 V) and reset (6.5 V) processes is observed for 10^4 cycles. **(C)** Size-dependent switching speed of PCRAM. Shorter pulse widths are achieved when the cell size is decreased for a fixed applied-voltage pulse (1 V). The pulse width decreases additionally when the incubation field (0.3 V) is applied, further improving the speed of PCRAM. **(D)** Minimum pulse width versus incubation field (voltage) for a PCRAM cell (300 nm). The pulse width reduces as the incubation field increases, revealing the incubation-dependent crystallization speed of PC materials.



Even faster crystallization is observed when the incubation field is applied to cells with smaller feature sizes. In general, PC materials are divided into two groups, depending on the crystallization mechanism. For nucleation-dominated crystallization, a large number of crystalline nuclei form in the amorphous region. For growth-dominated crystallization, the transformation of the amorphous region is directed by the growth of the crystalline phase from the crystalline rim surrounding the amorphous region/interface between the PC material and dielectric sidewalls in optical and electrical PC devices (8, 25, 26). Growth-dominated crystallization can be characterized by the strong dependence of crystallization time on the size of the amorphous/active region (in this case, cell size). This growth-dominance effect can be seen in Fig. 2C where, under no incubation field, the pulse width for set reduces from 70 to 10 ns as the cell size decreases from 300 to 50 nm. A very different effect is observed when an incubation field is applied. We found that the pulse width decreases even more substantially as the cell size decreases (by 28% at 300 nm and by 95% at 50 nm), suggesting a much faster nucleation and growth induced by a combination of size and incubation effects.

One of the key results of our study is the ability to control the crystallization speed (nuclea-

tion and growth time) by varying the intensity of the incubation field. This implies that nucleation and growth can be further accelerated via stronger incubation fields. Figure 2D shows the pulse width needed to set a cell (300 nm) at different incubation fields. We observed that the pulse width decreases from 70 to 50 ns as the incubation field increases (0 to 0.3 V), revealing the dependence of the crystallization speed of PC materials on the intensity of the incubation field. Previous works on GST (27) and Ag-In-Sb-Te (28, 29), using optical (laser pump-probe) stimulation and direct thermal-annealing treatment, have also shown that the crystal-nucleation rate can be manipulated. However, the crystallization times achieved in those studies were on the order of only several tens of nanoseconds to a few microseconds.

The microscopic origin of the ultrafast crystallization resulting from the application of an incubation field was investigated by performing ab initio molecular dynamics (AIMD) simulations for 180-atom models of GST (23). To simulate the crystallization process on the application of incubation fields and/or electrical pulses, two temperatures of 420 and 600 K were applied to the GST models to mimic the respective annealing/Joule-heating processes (hereafter, annealing at 420 K is referred to as “preannealing”) (fig. S6). The detailed procedures for simulating

the amorphous-to-crystal phase transition are described in fig. S7. For a more complete (statistical) analysis, we studied three amorphous (a-) models (models 1 to 3), which were obtained by independently quenching different configurations of liquid GST. The basic structural units of crystalline GST (that is, of the metastable distorted rock salt structure)—such as fourfold rings, planes, and cubes—were used to characterize the local structural order (or the crystallization process during annealing) (23).

To investigate the effects of applying an incubation field on the crystallization behavior, one of the a- models (3) was preannealed at 420 K for 270 ps and subsequently annealed at 600 K. For comparison, the same model was also annealed at only 600 K. In both cases, we examined the crystallization process by studying the evolution of the number of cubes; the onset time (t_o) of crystallization is defined here as the starting point of the increase of the stable cube cluster, whereas the end of crystallization is the point at which no further growth in the number of cubes is observed.

We observed much faster crystallization in the preannealed model, as shown in Fig. 3A. There is a large decrease in t_o for the preannealed model (~80 ps) compared with the model that was not preannealed (~350 ps). We also observed a similar shortening in t_o for another model that

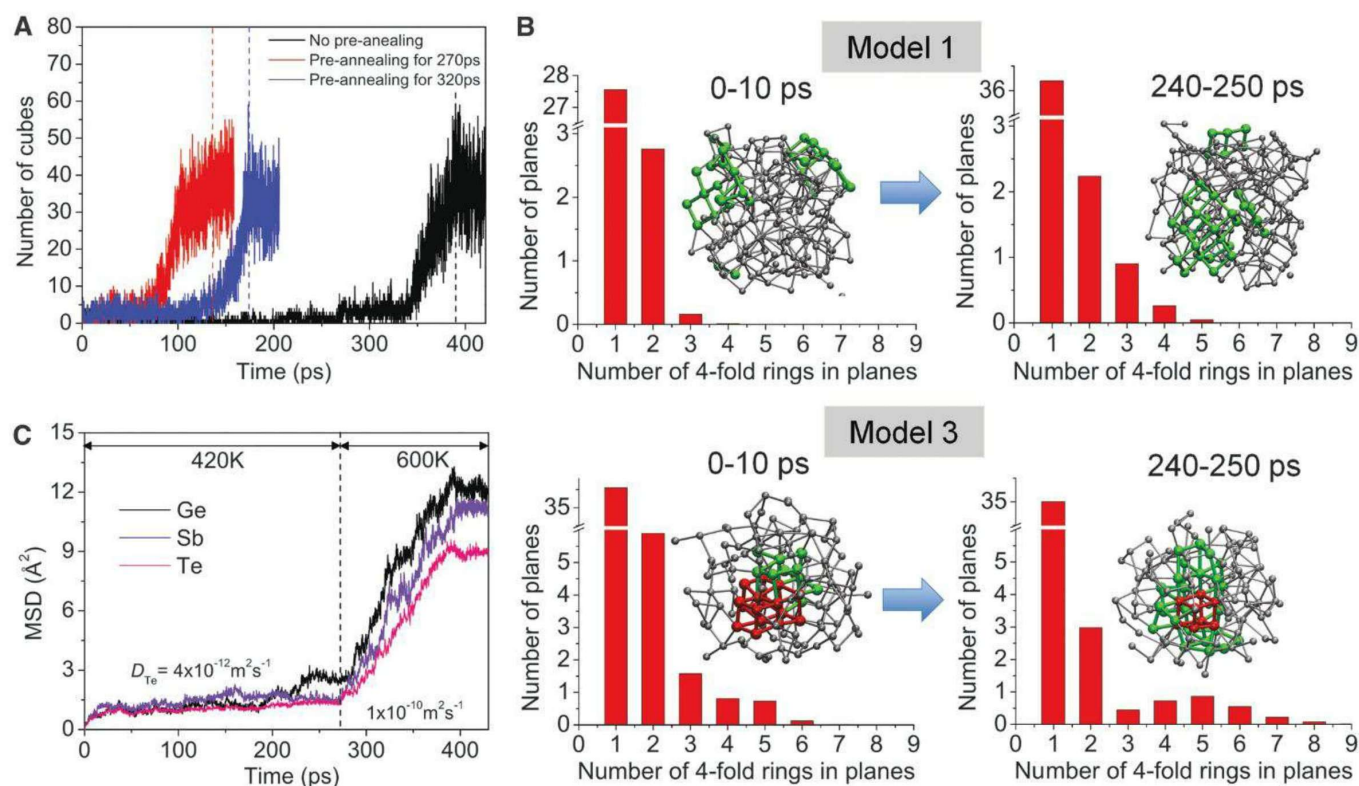


Fig. 3. AIMD simulations. (A) Variation of the number of cubes during annealing at 600 K with different preannealing times. (B) Population of fourfold rings and planes, composed of different numbers of fourfold rings, in model 1 before and after preannealing. The number of planes was averaged over the time interval denoted above each panel. A similar change in the distribution of

the number of planes is seen in model 3. Green atoms are in planes; red atoms are in cubes. (C) Mean-squared displacement data for each type of atom during two successive annealing steps at 420 and 600 K (model 3). Diffusion coefficients for Te atoms (D_{Te}), calculated at each annealing temperature, are shown as an example.

was preannealed for longer times. The corresponding changes in the atomic structures for these two different annealing routes are shown in Fig. 1A, which explains the origin of the faster crystallization. Before t_0 , there were substantial thermal fluctuations upon annealing at 600 K that resulted in the disruption of the initial cluster in the model that was not preannealed. After a period of repeated generation and annihilation of transient clusters (incubation time), a stable cluster (with a different orientation from the initial one) formed and grew to crystallize. This is not the case for the preannealed models; the initial ordered structure (further grown during preannealing) maintains its shape and grows along its original symmetry axis throughout the crystallization. Thus, the shortening in t_0 for the preannealed models is associated with shorter incubation times for nucleation and growth, which is triggered by the structural ordering during preannealing.

We found that the predominant structural-ordering mechanism is the formation and growth of clusters of planes of fourfold rings (i.e., a structural feature of the rocksalt structure of the crystalline phase of GST), as shown in Fig. 3B. An overall medium-range ordering in the amorphous phase (in terms of the bond angles and atomic distances with the second-nearest neighbors) is also revealed (fig. S8).

This prestructural ordering is stable in nature. Figure 3C shows the mean-squared displacements of atoms during the two-step annealing process. The diffusion coefficient at 420 K is estimated to be about two orders of magnitude smaller than at 600 K. Accordingly, the structural ordering in a-GST at 420 K was found to be diffusionless; the formation and/or growth of a cluster of planes occurs mostly via cooperative (bond-rotational) movements (over much less than interatomic distances) of a few relevant atoms, in direct contrast to the structural ordering at 600 K induced by bond-breaking diffusional processes (fig. S10). This difference in behavior is most likely due to insufficient thermal energy available at 420 K to overcome the energy barrier for diffusion. Consequently, the growth speed and the size of clusters at this low temperature should be kinetically limited, in spite of the driving force for nucleation (that is, free-energy difference) being greater than at higher temperatures (30).

Important observations from our simulations are as follows: (i) In general, annealing at low temperatures (at least at 420 K, which is much lower than the peak crystallization temperature) leads to medium-range structural ordering in a-GST, including the formation (and growth) of clusters (prestructural ordering). (ii) Prestructural ordering can induce faster crystallization via shorter incubation times for the nucleation upon subsequent annealing at higher temperatures.

The origin of the faster crystallization under the influence of the incubation field can be similarly understood with the help of the concept

of a cluster-size distribution, the basis of the kinetic model of nucleation (31, 32). Specifically, applying an incubation field induces a temperature increase (~ 100 K) by Joule heating. This elevated temperature is not high enough to activate spontaneous nucleation and growth; it simply leads to an overall structural ordering with the development of clusters in the amorphous matrix, which is important for the amorphous stability. Naturally, a new cluster-size distribution results at this temperature, where the population of larger clusters (mostly smaller than the critical nucleus size) becomes more abundant. A greater population of larger clusters (as well as overall medium-range ordering) then enhances the probability of successful nucleation (via shorter incubation times required for nucleation) and of subsequent growth, when an electrical pulse is applied, the energy of which is high enough to activate diffusion-induced structural ordering. Thus, the electrical controllability of the crystallization speed (as in Fig. 2D) is closely associated with the effect of temperature on the cluster-size distribution that determines the nucleation probability. On the atomic scale, such structural ordering, even at a low temperature, seems to be a general feature of PC materials, being enabled by the intrinsic electronic configurations of the constituent atoms, facilitating pure p -bonding (33).

The experimental and simulation findings show that ultrafast crystallization can be achieved by applying an incubation electrical field. The additional energy needed to apply the incubation field is much smaller than that for crystallization, as the applied incubation voltage is much less than the typical threshold-switching voltage (0.5 to 1 V). Furthermore, the incubation field can be applied after a reset process for a minimum time (fig. S11). This reduces the extra energy needed, in contrast to applying the incubation field consistently, while waiting for the set process in real device operations. To further reduce the energy required, PCRAM can also be operated from a lower resistance level (fig. S12).

In addition to showing rapid crystallization speeds, we also show the endurance of memory cells operating at such high speeds. This indicates that our approach is feasible for device applications.

These rapid crystallization times, as well as high amorphous-state stability, are achieved through an electrically induced incubation process in PCRAM devices that alters the crystallization kinetics of the a-GST. In principle, this method is applicable to all types of PC materials and memory-device structures, so that an appropriate combination of programming schemes and PC materials opens opportunities for optimizing PCRAM device performance.

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Acknowledgments: We acknowledge support by the Advanced Memory Research Program of A*STAR, the Nonvolatile Memory Research Program of Data Storage Institute (Singapore), and the Engineering and Physical Sciences Research Council (UK). The experiments were carried out in the Data Storage Institute, A*STAR, and the AIMD simulations were performed using the Cambridge High-Performance Computing Facility. D.L. thanks the NUS Graduate School for Integrative Sciences and Engineering for scholarship support.

Supplementary Materials

www.sciencemag.org/cgi/content/full/336/6088/1566/DC1
Materials and Methods
Figs. S1 to S12
References (34–44)

6 March 2012; accepted 10 April 2012
10.1126/science.1221561

Roton-Type Mode Softening in a Quantum Gas with Cavity-Mediated Long-Range Interactions

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Long-range interactions in quantum gases are predicted to give rise to an excitation spectrum of roton character, similar to that observed in superfluid helium. We investigated the excitation spectrum of a Bose-Einstein condensate with cavity-mediated long-range interactions, which couple all particles to each other. Increasing the strength of the interaction leads to a softening of an excitation mode at a finite momentum, preceding a superfluid-to-supersolid phase transition. We used a variant of Bragg spectroscopy to study the mode softening across the phase transition. The measured spectrum was in very good agreement with *ab initio* calculations and, at the phase transition, a diverging susceptibility was observed. The work paves the way toward quantum simulation of long-range interacting many-body systems.

Experiments with ultracold gases have succeeded in realizing a variety of quantum many-body phases by exploiting the tunability of short-range atom-atom interactions (1). Creating quantum gases with long-range interactions (2–5) is motivated by the prospect of observing previously unexplored phenomena and phases (6). In particular, long-range interactions in a Bose-Einstein condensate (BEC) have been predicted (7–10) to give rise to an excitation spectrum similar to the roton spectrum observed in superfluid helium (11). A roton spectrum emerges from density-density correlations that can be induced by short-range van der Waals interactions, as in liquid helium, or by momentum-dependent long-range interactions in dilute quantum gases (7). Such mode softening at finite momentum has been proposed as a possible route to a supersolid phase (12).

Important steps toward realizing quantum gases with long-range interactions have been achieved by cooling atomic species with large magnetic dipole moments to quantum degeneracy (2, 5) and by efforts to prepare ultracold polar molecules in their ground state (3, 4). Effects of long-range interactions in quantum gases have been observed in the anisotropic expansion, in collective as well as Bloch oscillations, and in the stability of interacting dipolar BECs (13–16). However, a roton-type mode softening requires very strong long-range interactions and is challenging to observe in quantum gases.

A different approach to long-range interactions in cold gases makes use of the radiative coupling between electric dipoles induced by off-resonant laser light, which has been shown theoretically to cause a roton-like minimum in the energy spectrum of a BEC (8, 10). A large enhancement of such radiative coupling is achieved by placing the dipoles into an optical resonator, which leads to strong global atom-atom interactions (17–20).

We create long-range interactions in a BEC by placing it into an optical high-finesse cavity and irradiating the atoms with a transverse pump laser, which is far detuned from the atomic transition frequency (21–23) (Fig. 1A). The induced electric dipoles of the atoms oscillate at the pump laser frequency and collectively couple to a single cavity mode, which is detuned from the pump frequency by a few cavity linewidths. In turn, the induced cavity field leads to an ac Stark shift in the atoms. The resulting atom-atom interaction extends over the entire atomic cloud and is tunable in strength. For a critical strength of the long-range interactions, the BEC undergoes a quantum phase transition to a supersolid phase with checkerboard density order (22), as observed in (23). At the phase transition, a discrete spatial symmetry provided by the cavity mode structure is broken, thus establishing nontrivial diagonal long-range order. We have developed a method

to measure the excitation spectrum across this phase transition. The method combines Bragg spectroscopy (24) and cavity-enhanced Bragg scattering, and we use it to observe a notable change in the dispersion relation while crossing the phase transition. This is complemented by a diverging susceptibility, in agreement with the second-order nature of the phase transition (Fig. 1B).

The cavity-mediated long-range interaction is described by the Hamiltonian $\hat{H}_{aa} = \int \hat{\Psi}^\dagger(\mathbf{r}) \hat{\Psi}^\dagger(\mathbf{r}') \mathcal{V}(\mathbf{r}, \mathbf{r}') \hat{\Psi}(\mathbf{r}') \hat{\Psi}(\mathbf{r}) d^3\mathbf{r} d^3\mathbf{r}'$ with atomic field operator $\hat{\Psi}(\mathbf{r})$. This Hamiltonian is obtained from the dispersive atom-light interaction Hamiltonian by adiabatically eliminating the fast cavity field dynamics (19, 25). The interaction potential has the form

$$\mathcal{V}(\mathbf{r}, \mathbf{r}') = V \cos(kx) \cos(kz) \cos(kx') \cos(kz') \quad (1)$$

and describes the ac Stark shift experienced by an atom at position $\mathbf{r}$ as a result of the cavity field induced by a second atom at position $\mathbf{r}'$. As a consequence of the interference between the mediating cavity field and the transverse pump field, the spatial dependence of the interaction is determined by the corresponding mode functions $\cos(kx)$ and $\cos(kz)$, where $k = 2\pi/\lambda$ denotes the optical wave vector (Fig. 1A). The interaction strength $V = \hbar\eta^2/\tilde{\Delta}_c$ (where $\hbar$ is the Planck constant divided by 2π) depends on the two-photon Rabi frequency η , which can be tuned experimentally via the transverse pump power P (25). The sign of V is determined by the detuning $\tilde{\Delta}_c = \omega_p - \tilde{\omega}_c$ between the transverse pump laser frequency ω_p and the dispersively shifted cavity resonance $\tilde{\omega}_c$. In the experiment, $|\tilde{\Delta}_c|$ is large relative to the cavity linewidth 2κ .

For negative V , the cavity-mediated interaction induces density correlations in the atomic

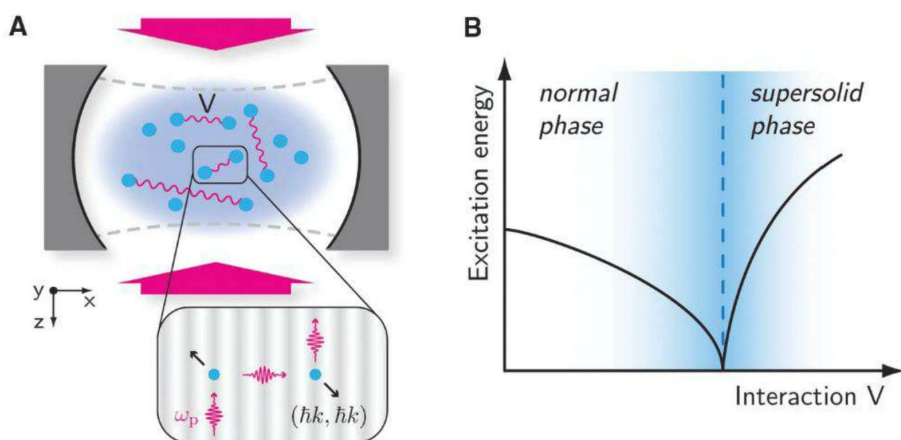


Fig. 1. Experimental scheme and mode softening. **(A)** A ^{87}Rb BEC inside a Fabry-Perot resonator is transversally illuminated by a far red-detuned standing-wave laser field. In a quantized picture, atoms off-resonantly scatter photons from the pump field into a close-detuned TEM_{00} cavity mode and back at a rate $NV/\hbar$ (see text), creating and annihilating pairs of atoms in the superposition of momenta $(p_x, p_z) = (\pm\hbar k, \pm\hbar k)$ (one of four possible processes is shown schematically). This results in global interactions between all N atoms. The interaction strength V is controlled via the power of the transverse laser field. **(B)** The cavity-mediated atom-atom interaction causes a softening of a collective excitation mode at momenta $(\pm\hbar k, \pm\hbar k)$ and a diverging susceptibility (blue shade) at a critical interaction strength (dashed line).

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Fig. 2. Probing the excitation spectrum in the normal phase ($P = 0.65P_{cr}$). **(A)** Absorption image of the atomic cloud after probing the cavity for 0.5 ms and subsequent ballistic expansion over 7 ms. **(B)** Excited atomic population N_e with momenta ($\pm\hbar k, \pm\hbar k$), deduced from the regions enclosed by blue circles in (A), as a function of the probe-pump detuning δ (circles). The solid line shows a fit based on the theoretical model (see text). The background in N_e originates from a residual thermal component extending into the detection region. **(C)** Recorded (circles) and fitted (solid line) mean intracavity photon number n_{ph} during probing. The dashed line indicates the intracavity photon number of the probe pulse, obtained in the absence of atoms. **(D)** Temporal evolution of $N_e(t)$ inferred theoretically from the fit shown in (C). **(E)** Mean intracavity photon number $\langle n_{ph} \rangle$, averaged over the probe interval, as a function of δ (circles). The fit (solid line) takes into account the calibrated photon number of the probe pulse (dashed line). From the recorded photon signal, we deduce the photon number originating from Bragg scattering off the created excitations (dashed-dotted line). The shading in (B) and (E) indicates the fluctuations caused by variations of the relative phase ϕ between different experimental runs. The detuning of the pump beam from the empty cavity resonance was $\Delta_c = -2\pi \times 18$ MHz; total atom number $N = 1.6 (\pm 0.2) \times 10^5$. The damping constant γ was set to $2\pi \times 0.6$ kHz (see text).

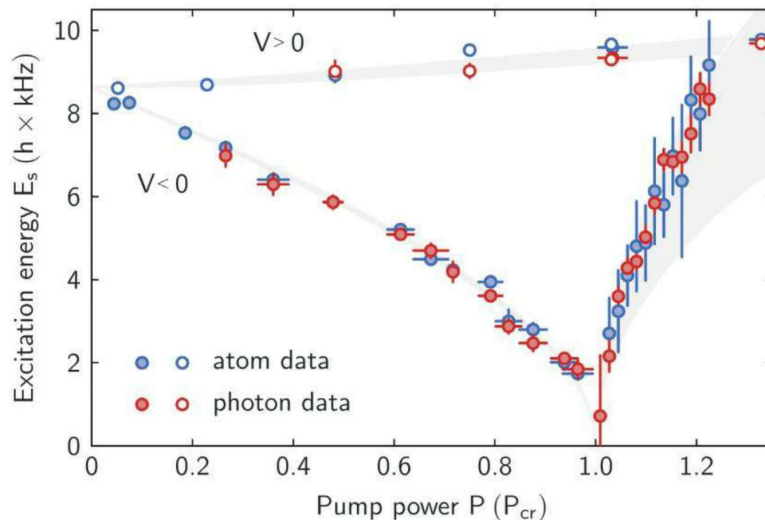
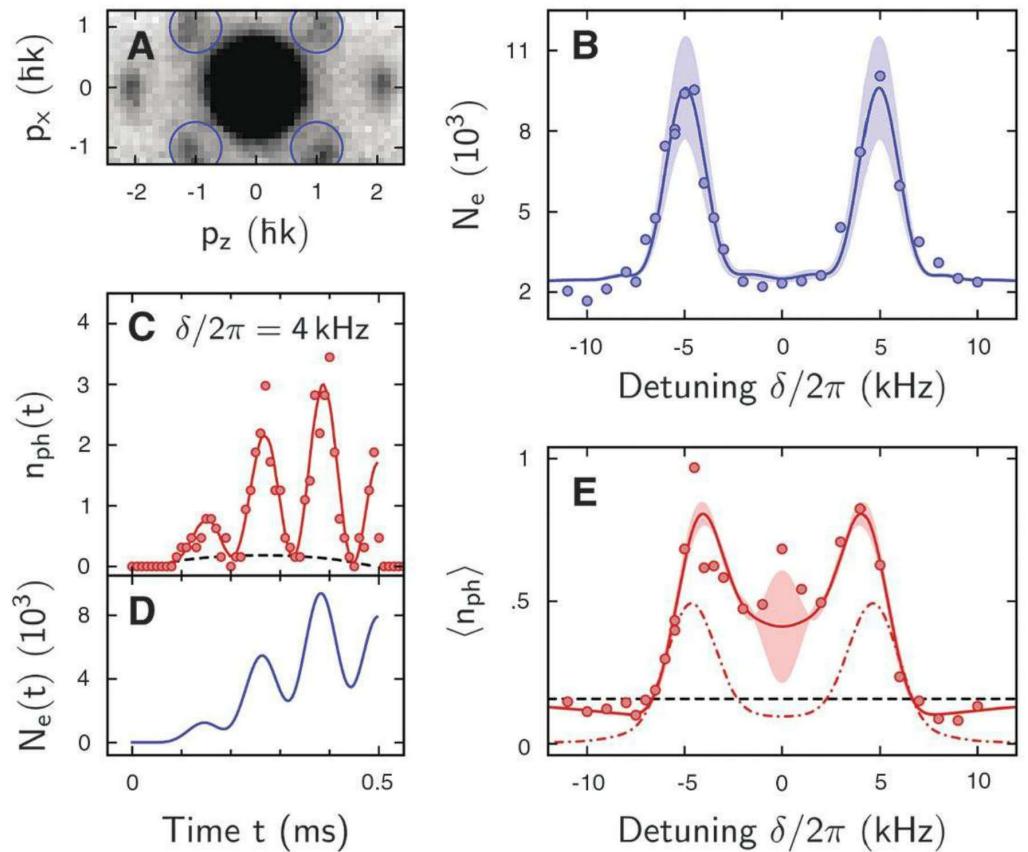


Fig. 3. Excitation spectrum. Measured resonance frequencies $E_s/\hbar$, obtained from atomic (N_e) and photonic ($\langle n_{ph} \rangle$) signals, are shown in blue and red, respectively, for positive (open circles) and negative (solid circles) interaction strength V . Gray shading shows the theoretical prediction including experimental uncertainties (25). The experimental parameters are $N = 1.7 (\pm 0.2) \times 10^5$, $\Delta_c = -2\pi \times (19.8, 23.2)$ MHz for $V < 0$ (normal and supersolid phase), and $\Delta_c = +2\pi \times 15.1$ MHz for $V > 0$. For $V > 0$, the pump power is scaled in terms of the critical pump power observed for $\Delta_c = -2\pi \times 19.7$ MHz.

cloud with spatial periodicity of λ along the pump and cavity direction. In momentum space, this corresponds to the creation and annihilation of

pairs of correlated atoms in the momentum mode $|e\rangle$, which is the symmetric superposition of the four momentum states $|p_x, p_z\rangle = |\pm\hbar k, \pm\hbar k\rangle$. For

a macroscopically populated zero-momentum mode containing N atoms, this is described by the Hamiltonian

$$\hat{H} = 2E_r \hat{c}^\dagger \hat{c} + \frac{NV}{4} (\hat{c}^\dagger + \hat{c})^2 \quad (2)$$

(25), where $\hat{c}^\dagger$ creates particles in mode $|e\rangle$. The first term in Eq. 2 corresponds to the kinetic energy of atoms in mode $|e\rangle$, with recoil energy $E_r = \hbar^2 k^2 / 2m$ and atomic mass m .

The elementary excitations of the Hamiltonian $\hat{H}$ are of a collective nature, and their eigenenergy E_s softens for increasing interaction strength V (Fig. 1B). The excitation energy E_s vanishes at a critical interaction strength V_{cr} where interaction and kinetic energy are balanced—that is, $N|V_{cr}| = 2E_r$. This marks the transition point between a normal and a supersolid phase. In the supersolid phase, a macroscopic occupation of the mode $|e\rangle$ gives rise to a checkerboard pattern in the atomic density distribution, which is accompanied by the buildup of a coherent cavity field amplitude a_0 . The energy of collective excitations rises again with increasing interaction strength and approaches the single-particle excitation energy of the induced optical checkerboard potential.

To measure the excitation spectrum of the system at momenta $(\pm\hbar k, \pm\hbar k)$, we perform a

variant of Bragg spectroscopy (24, 26). After preparing the system at a given interaction strength, the cavity field is excited with a weak probe pulse along the x axis (Fig. 1A). Interference between the cavity probe field and the transverse pump field results in an amplitude-modulated lattice potential $\hbar\eta\sqrt{n_{\text{pr}}}\cos(\delta t + \varphi)\cos(kx)\cos(kz)$, with probe-pump detuning $\delta = \omega_{\text{pr}} - \omega_p$, relative phase φ , and mean intracavity photon number n_{pr} of the probe field. In momentum space, this corresponds to the perturbation $\hat{H}_{\text{pr}} = \hbar\eta\sqrt{n_{\text{pr}}}\cos(\delta t + \varphi)(\hat{c}^\dagger + \hat{c})$. After probing, all laser fields are switched off. This projects the created collective excitations onto the free-space momentum states $|\pm\hbar k, \pm\hbar k\rangle$, which are detected via absorption imaging subsequent to ballistic expansion (Fig. 2A). A typical resonance curve of the excited momentum state population $N_e = \langle \hat{c}^\dagger \hat{c} \rangle$ as a function of δ is shown in Fig. 2B. Clear resonances are revealed both for positive and negative probe-pump detuning, corresponding to stimulated scattering of probe photons into the pump field and vice versa. The corresponding resonance frequency $E_s/\hbar$ is obtained from a fit (solid line) based on the model described by Eq. 2 (25).

The measured excitation spectrum as a function of pump power P is displayed in Fig. 3. When increasing the interaction strength in the normal phase toward the critical point, the excitation energy E_s exhibits a distinct softening. In contrast, for positive V , the excitation gap is observed to increase with interaction strength in accordance with the absence of a phase transition. The increasing gap reflects the tendency of the cavity-mediated interactions to suppress density fluctuations with λ periodicity.

We monitor the cavity output light on a single-photon counting module. This gives us real-time access to the formation of collective excitations during probing. Because of matter-wave interference between the ground-state component of the condensate and the created excitations, a spatial density modulation evolves in time. This density modulation leads to Bragg scattering of transverse pump laser light into the cavity mode. The output from the cavity therefore follows the oscillatory evolution of this density modulation (Fig. 2C). The total intracavity photon number, averaged over the probe interval, again exhibits a double resonance (Fig. 2E) whose characteristic shape has its origin in interference between the probe field and the Bragg-scattered pump field. This provides a second method to determine the resonance frequency $E_s/\hbar$, as shown in Fig. 3.

We model the dynamics of the system during probing by evolving the Hamiltonian $\hat{H} + \hat{H}_{\text{pr}}$ in time, taking into account the recorded shape of the probe pulse (see Fig. 2C). Quantitative agreement with the data is obtained by including a phenomenological damping rate γ in the time evolution of $\hat{c}$ (25). The corresponding time evolution of the momentum population $N_e(t)$ is shown in Fig. 2D. Depending on the relative phase φ , which is not controlled in the experi-

ment, the phase of the excited density oscillation varies between different experimental runs. This leads to an intrinsic fluctuation of the quantities N_e and $\langle n_{\text{ph}} \rangle$, as indicated by the shaded areas in Fig. 2, B and E.

In the supersolid phase, the perturbation creates excitations on top of the macroscopic steady-state population in momentum mode $|e\rangle$ (23). Because of matter-wave interference, the detected population N_e is strongly affected by fluctuations of the relative phase φ . Yet the variance of N_e around the steady-state value, deduced from several experimental runs, shows clear resonances. Alternatively, we use the oscillation amplitude of the intracavity photon number around the steady-state value $|\alpha_0|^2$ to extract the excitation energy E_s (25). As shown in Fig. 3, the excitation energies deduced from these two signals rise again with pump power P and provide a consistent picture.

The observed excitation spectrum (Fig. 3) is in qualitative agreement with a soft mode energy $E_s = 2E_r\sqrt{1 + V/|V_{\text{cr}}|}$, which follows from Eq. 2. Quantitative agreement is obtained by further taking into account the lattice potential of the transverse pump beam as well as contact interactions between colliding atoms (25). Accordingly, the single-particle mode $|e\rangle$ in Eq. 2 is replaced by a Bogoliubov mode $|1\rangle$ and the interaction element of $V(\mathbf{r}, \mathbf{r}')$ between the condensate mode and the excited state $|1\rangle$. In the normal phase, the state $|1\rangle$ lies in the lowest-energy band of the transverse lattice potential with quasi-momentum $(\pm\hbar k, \pm\hbar k)$ and bare energy E_1 . For $P = 0$, the excitation energy E_s reaches the Bogoliubov energy $E_1 = \hbar \times 8.7$ kHz, which is shifted by the mean-field energy with respect to the kinetic en-

ergy $2E_r = \hbar \times 7.5$ kHz. In the supersolid phase, the Bogoliubov mode $|1\rangle$, which is dominantly affected by the long-range interactions, lies in a higher-energy band at zero quasi-momentum of the emerging checkerboard lattice potential. As a result of competition between the increasing band energy and the renormalized interaction energy, the excitation energy E_s rises again.

Another signature for a softened dispersion relation at finite momentum is provided by the susceptibility to external density perturbations. In terms of the static structure factor $S(k)$, which measures the density-density response, this is expressed by Feynman's relation $S(k) = (\hbar k^2/2m)/\omega(k)$, where $\omega(k)$ denotes the dispersion of a homogeneous system (27). To determine the density response in our system, we quantify the amount of pump light that is Bragg-scattered into the cavity. This allows us to measure the probe-induced density modulation $\langle \delta \hat{\rho}_e \rangle$, where $\delta \hat{\rho}_e$ denotes the density fluctuation operator at momenta $(\pm\hbar k, \pm\hbar k)$. We define the corresponding quadratic density response function $\mathcal{R}_\rho$ as the spectral weight of $\langle \delta \hat{\rho}_e \rangle^2$, normalized to the pump power P and the probe pulse area $\langle n_{\text{pr}} \rangle$. The response function $\mathcal{R}_N$ of the atomic population N_e in the excited momentum state $|e\rangle$ is defined in a similar way (25).

Approaching the critical point from below, a strongly increasing density response is observed (Fig. 4). This indicates the presence of enhanced density fluctuations with λ -periodic correlations in the unperturbed system. The data are in good agreement with our model (shaded area in Fig. 4), which predicts $\mathcal{R}_\rho$ to scale as $(E_1/E_s)^2$ in the normal phase, in accordance with Feynman's relation. In the supersolid phase, the density response decreases again as a function of P , as expected for the increasing excitation energy

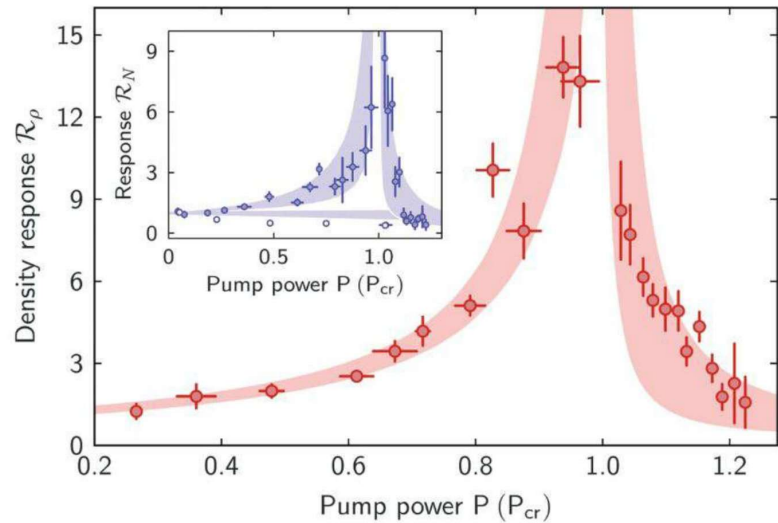


Fig. 4. Response to density perturbations. Shown is the density response in the normal and supersolid phases, normalized to the noninteracting case ($P = 0$). The shaded areas show the theoretical prediction including experimental uncertainties and fluctuations caused by the uncontrolled relative phase φ . The atomic damping constant $\gamma = 2\pi \times 0.5 (\pm 0.1)$ kHz was obtained from the fit to the data in the normal phase. The inset shows the response of the excited momentum state population N_e for negative (solid circles) and positive (open circles) interaction strength V . The experimental parameters were the same as in Fig. 3.

(Fig. 3). A similar behavior is observed for the response in the momentum state population N_e (Fig. 4, inset), which exhibits a larger sensitivity to variations of the relative phase φ (25). For positive interaction V (Fig. 4, inset), the response of the system is reduced with respect to the non-interacting case, $V = 0$.

We have observed a roton-type mode softening causing a superfluid-to-supersolid transition in a model system for long-range interactions. Increasingly complex spatial structures of long-range atom-atom interactions (28–30) can be tailored by extending the experimental setup to multiple cavity modes.

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Acknowledgments: We thank H. Tureci, P. Domokos, and H. Ritsch for stimulating discussions. Supported by Synthetic Quantum Many-Body Systems (European Research Council advanced grant), Nanodesigning of Atomic and Molecular Quantum Matter (European Union, Future and Emerging Technologies open), National Centre of Competence in Research/Quantum Science and Technology, and the European Science Foundation (POLATOM).

Supplementary Materials

www.sciencemag.org/cgi/content/full/science.1220314/DC1

Supplementary Text

Fig. S1

References (31–40)

9 February 2012; accepted 2 May 2012

Published online 17 May 2012;

10.1126/science.1220314

Baseline Map of Carbon Emissions from Deforestation in Tropical Regions

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Policies to reduce emissions from deforestation would benefit from clearly derived, spatially explicit, statistically bounded estimates of carbon emissions. Existing efforts derive carbon impacts of land-use change using broad assumptions, unreliable data, or both. We improve on this approach using satellite observations of gross forest cover loss and a map of forest carbon stocks to estimate gross carbon emissions across tropical regions between 2000 and 2005 as 0.81 petagram of carbon per year, with a 90% prediction interval of 0.57 to 1.22 petagrams of carbon per year. This estimate is 25 to 50% of recently published estimates. By systematically matching areas of forest loss with their carbon stocks before clearing, these results serve as a more accurate benchmark for monitoring global progress on reducing emissions from deforestation.

Although carbon emissions from fossil fuel use are relatively well quantified, emissions from land-use change—one of the largest anthropogenic sources of carbon to the atmosphere globally—are the most uncertain component of the global carbon cycle (1). The magnitude of these emissions has remained poorly constrained because of the use of different data, assumptions, and methodologies for estimating

rates of deforestation, carbon stocks in vegetation and soils (2), the mode of clearing carbon, the fate of the cleared carbon, the response of the soil carbon pool to deforestation, and lag effects from historical land cover change (3).

Existing studies of the carbon balance of land-use change in tropical regions (4–7) incorporate carbon emissions from net changes in forest area, emissions from timber harvesting, and carbon removals from forest regrowth after abandonment. Statistics on net forest area changes and rates of timber harvesting, as reported by countries to the United Nations Food and Agriculture Organization (FAO), and a carbon cycle “bookkeeping” model were used to estimate net carbon emissions from the tropics as 1.9 and 2.2 petagrams of carbon per year (Pg C yr^{-1}) for the 1980s and 1990s, respectively (7). Data reported to FAO are known to be unreliable (8), and the bookkeeping

model uses many broad assumptions about the fate of cleared lands and their respective carbon stocks to estimate the associated net carbon impacts. The emergence and widespread use of multiresolution remote sensing imagery to track land-cover change resulted in new, lower estimates of net carbon emissions for the 1990s of 0.9 and 1.1 Pg C yr^{-1} (9, 10), caused mainly from the use of different estimates of tropical deforestation rates. Recent studies (11, 12) for the 2000s that use the bookkeeping model estimate average net emissions from tropical land-use change as 1.3 and 1.0 Pg C yr^{-1} (table S1).

Although these studies are useful for understanding the role of tropical land-use change in the global carbon cycle, a policy mechanism that proposes to compensate developing countries for reducing emissions from deforestation and forest degradation (REDD) will benefit from estimates of emissions from gross deforestation that are disaggregated from the forest regrowth term and that do not use a priori assumptions about the fate of vegetation carbon stocks after clearing (4–7, 9–12). According to the FAO data and the bookkeeping model, gross emissions from tropical deforestation, without the inclusion of forest regrowth, are reported to be $2.8 \pm 0.5 \text{ Pg C yr}^{-1}$ for the period 2000 to 2007 and 2.2 Pg C yr^{-1} for the period 2000 to 2010 (table S1) (11, 12). However, to inform ongoing policy discussions, more transparent and spatially explicit estimates of emissions are needed in which data for forest area loss and carbon stocks are assessed independently and the areas of forest loss are matched with the carbon stocks of the forests undergoing conversion.

Global loss in gross forest cover from 2000 to 2005 (13, 14) and the spatial distribution of forest carbon stocks in tropical regions for the period circa 2000 (15) have been quantified by using

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multiscale remote sensing techniques, but they have not been used to estimate corresponding carbon emissions. We combined these data for 75 developing countries across three continental regions (Latin America, sub-Saharan Africa, and South and Southeast Asia) to develop a systematic, spatially consistent, and transparent estimate of gross carbon emissions from deforestation between 2000 and 2005 (16). We define deforestation as the area of forest cover removed because of any human or naturally induced disturbance (16). We use political rather than biome boundaries to define our study region (fig. S1) so as to provide consistent country-specific reference levels

that can serve as a preliminary basis for emission reduction targets. Forest carbon stock estimates are partitioned into above- and belowground carbon density [megagrams of carbon per hectare (Mg C ha^{-1})] at 1-km spatial resolution, but the forest loss estimate is expressed in terms of the total area lost in 18.5-by-18.5-km blocks globally. We resolve the disparate spatial resolution of these two maps by repeating a randomization procedure in which forested 1-km pixels within a given 18.5-km block are selected randomly ($n = 1000$ realizations) until the forest loss quota (in hectares) for the block is met. The total carbon values of selected 1-km pixels (in megagrams of

carbon) are then summed across the block to derive an emissions estimate. The average of the 1000 estimates associated with forest loss is assigned as the best estimate of emissions per 18.5-km block. By colocating biomass density data with forest loss data at relatively high spatial resolution, we improve on prior approaches that aggregate data at much coarser, regional scales (12). Additionally, a complete statistical uncertainty analysis around our final emissions estimate per block is generated by using a Monte Carlo-style sampling simulation that incorporates the uncertainty in above- and belowground carbon estimates at the 1-km scale and the uncertainty in the forest loss estimates at the 18.5-km scale (fig. S2). This method of quantifying uncertainty in carbon emissions is also an improvement on previous attempts that rely mainly on qualitative estimates based on expert opinion (4–7, 9–12).

We estimated gross emissions resulting from gross loss in forest cover across the study region between 2000 and 2005 as $0.81 \text{ Pg C yr}^{-1}$, with a 90% prediction interval ranging from 0.57 to $1.22 \text{ Pg C yr}^{-1}$. This comprises 7 to 14% of total global anthropogenic CO_2 emissions over the time period analyzed. Forest loss in Latin America accounted for 54% of the total deforestation emissions, followed by 32% in South and Southeast Asia and 14% in sub-Saharan Africa. Our gross emissions estimate for tropical regions is approximately 25 to 50% of those reported previously for an overlapping time period by using the FAO/bookkeeping approach (Fig. 1) (11, 12).

We reviewed the main sources of uncertainty in our analysis and conclude that the errors in estimating forest loss in tropical regions from coarse resolution satellite data and calibrated with higher resolution Landsat data (13, 14) contributes ~5 to 10 times more to the total emissions uncertainty than the errors in estimates of forest

Fig. 1. Gross annual carbon emissions resulting from gross forest cover loss and peat drainage and burning between 2000 and 2005 compared with recently published estimates by (11) and (12) for an overlapping time period (2000–2007 and 2000–2010, respectively). Error bars represent 90% prediction intervals around median deforestation emission estimates. The estimates from (11) and (12) do not include peat emissions.

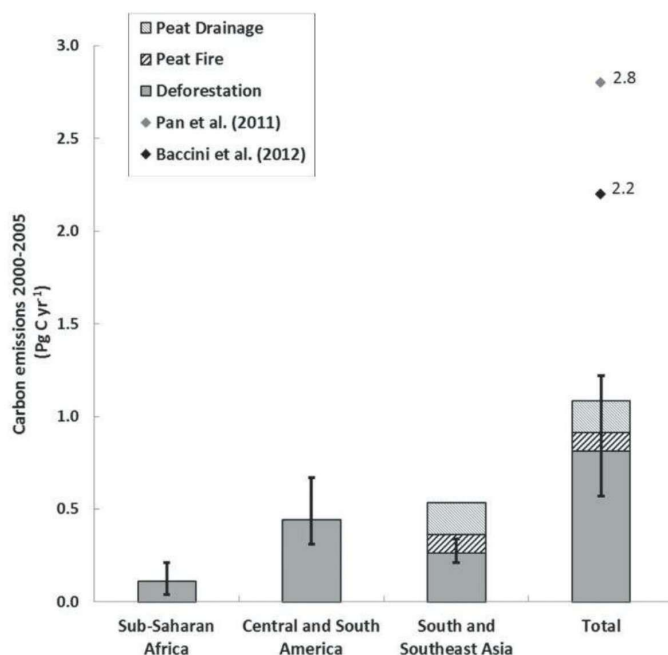


Table 1. Top carbon emitters from gross forest cover loss per region, 2000–2005. Countries are listed in order of highest to lowest carbon emissions between 2000 and 2005. Forest area and area loss values are based on (14), and average forest carbon density values are derived from (15).

Region	Country	Forest area 2000 (Mha)	Gross forest cover loss, 2000–2005 (Mha year ⁻¹)	Average forest carbon density (Mg C ha ⁻¹)	Carbon emissions, 2000–2005 (Tg C year ⁻¹)
Latin America and Caribbean	Brazil	458	3292	116	340
	Colombia	63	137	138	14
	Bolivia*	61	129	90	11
	Argentina*	49	437	24	10
	Venezuela*	49	115	134	9
Sub-Saharan Africa	Democratic Republic of the Congo	167	203	128	23
	Mozambique*	34	196	42	9
	Tanzania*	23	149	45	7
	Zambia	29	134	43	7
	Cameroon	26	54	142	7
South and Southeast Asia	Indonesia	107	701	155	105
	Malaysia	22	233	179	41
	Myanmar	33	186	155	29
	India	42	206	104	18
	Thailand	17	134	126	16

*Emission estimates (at 90% confidence) include potential emission values of zero in the uncertainty range.

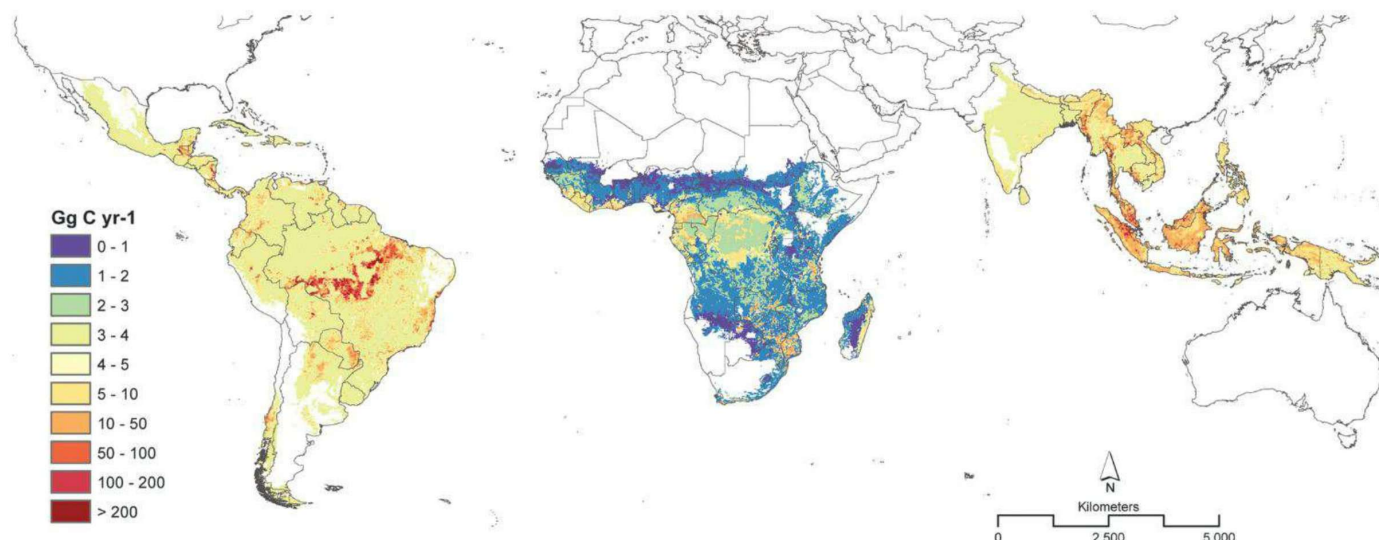


Fig. 2. Distribution of annual carbon emissions from gross forest cover loss between 2000 and 2005 mapped at a spatial resolution of 18.5 km.

carbon derived from aboveground biomass (15) and from the relationship between above- and belowground biomass (17), respectively. We focused our analysis on gross rather than net emissions because we can generate clear, statistically based uncertainty bounds around these estimates with the data and methods that are currently available. Net emission estimates require assumptions about the fate of converted lands to determine net carbon impacts that currently cannot be ascertained with statistical confidence across large regions. If robust data about the fate of converted lands existed and were used to generate a net emissions estimate, the result would be a reduction in our estimates of gross carbon emissions from deforestation shown in Fig. 1 (16).

Two countries—Brazil and Indonesia—produced the highest emissions between 2000 and 2005 and accounted for 55% of total emissions from tropical deforestation (Table 1). We present a map at the scale of analysis (18.5 by 18.5 km) (Fig. 2) to show the spatial distribution of emissions from tropical deforestation that captures the integrated importance of the extent of deforestation and the quantity of forest carbon stocks at regional scales. The relative importance of tropical Asia and Central and South America compared with tropical Africa as major sources of carbon to the atmosphere is shown in Fig. 2. Nearly 40% of total forest loss between 2000 and 2005 in our study region was concentrated in the dry tropics, but these losses accounted for only 17% of total carbon emissions, reflecting the low carbon density of these forests compared with tropical moist forests. Emissions are high in the Brazilian Amazon, but other areas of high emissions include Peninsular Malaysia, Laos, Sarawak (Malaysia), and Sumatra and Kalimantan (Indonesia) in Southeast Asia and, to a lesser extent, the Congo Basin in Africa.

The forest cover loss data used to derive the emissions estimates were generated by using

a procedure based on a regression estimator (13, 14) designed to produce aggregate estimates with reduced uncertainty for biomes, regions, and larger countries. Because forest loss estimates for individual 18.5-km blocks have relatively high uncertainty, we computed emissions at the aggregated country scale with less uncertainty (table S2) and present a relative ordering of emissions and relative uncertainty in emissions estimates by country (fig. S3). Our results for any 18.5-km block can be uncertain, but we are confident that the uncertainty estimation accurately bounds actual emissions at national and continental scales.

Our emission estimates reported above include those only from above- and belowground biomass carbon pools that generally account for 70 to 90% of total forest biomass carbon (18) and that we assume to occur immediately at the time of clearing. Other approaches (4–7, 9–12) include the soil carbon pool and consider the carbon stocks in replacement vegetation because soil emissions can result when land is cleared and converted to cultivated crops, and emissions from deforestation could be partially offset by carbon accumulation in regrowing vegetation. Spatially explicit data exist for estimating soil carbon stocks and for identifying the replacement land use (19, 20), but their precision is low, and the fate of the cleared land is difficult to ascertain accurately over large regions. Using standard methodologies (21), we conclude that accounting for soil carbon emissions increases our emissions estimate by less than 5%, and accounting for carbon accumulation in the replacement vegetation decreases our estimate by 5%, both of which fall within the uncertainty bounds of our analysis. Peat emissions are excluded from most estimates of emissions from land-use change (4–7, 9–12), although peat drainage and burning in Southeast Asia have been shown to contribute a substantial

portion of carbon emissions in past decades (22). Emissions from peat fire (23) and peat drainage (24) add another 0.099 and 0.173 Pg C yr⁻¹, respectively, which increases our total emissions estimate by approximately 25% but keeps the estimate within the uncertainty bounds of our analysis (Fig. 1).

Using the best available spatially consistent data sets on forest loss and forest carbon stocks, we have matched areas of forest loss with their carbon stocks before clearing to more accurately quantify gross emissions from deforestation in tropical regions. Our estimate is approximately 30% of previously published estimates for an overlapping time period. The largest source of uncertainty in our analysis is the estimates of gross forest-cover loss across large regions. This uncertainty in forest loss can be reduced through the detailed analysis of higher-resolution remotely sensed data. As developing countries collect and analyze new data to develop their reference levels for participation in a REDD mechanism, these data can be used to develop more accurate and precise estimates of country-scale emissions from all changes in forest cover. However, the current analysis will be useful as a benchmark against which progress on reducing emissions from deforestation may be assessed globally.

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Acknowledgments: Funding for this work was provided to Winrock International under contract 7150484 by the World Bank's World Development Report 2010: Development and Climate Change. The findings, interpretations, and conclusions expressed in this paper are entirely those of the authors. They do not necessarily represent the views of the World Bank and its affiliated organizations or those of the Executive Directors of the World Bank or the governments they represent. Support for the forest cover loss mapping work

was provided by National Aeronautics and Space Administration's Land Cover and Land Use Change and MEASURES programs under grants NNG06GD95G and NNX08AP33A. The authors would like to thank K. Mokany for providing the original data used to derive relationships between above- and belowground biomass. Forest loss data are available at <http://globalmonitoring.sdstate.edu/projects/gfm>. Carbon stock data are available at <http://carbon.jpl.nasa.gov>. Emissions data are available at www.appliedgeosolutions.com/science-paper.html.

Supplementary Materials

www.sciencemag.org/cgi/content/full/336/6088/1573/DC1
Materials and Methods
Figs. S1 to S3
Tables S1 and S2
References (25–29)

15 December 2011; accepted 3 May 2012
10.1126/science.1217962

Endophytic Insect-Parasitic Fungi Translocate Nitrogen Directly from Insects to Plants

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Most plants obtain nitrogen through nitrogen-fixing bacteria and microbial decomposition of plant and animal material. Many vascular plants are able to form close symbiotic associations with endophytic fungi. *Metarhizium* is a common plant endophyte found in a large number of ecosystems. This abundant soil fungus is also a pathogen to a large number of insects, which are a source of nitrogen. It is possible that the endophytic capability and insect pathogenicity of *Metarhizium* are coupled to provide an active method of nitrogen transfer to plant hosts via fungal mycelia. We used soil microcosms to test the ability of *M. robertsii* to translocate insect-derived nitrogen to plants. Insects were injected with ¹⁵N-labeled nitrogen, and we tracked the incorporation of ¹⁵N into amino acids in two plant species, haricot bean (*Phaseolus vulgaris*) and switchgrass (*Panicum virgatum*), in the presence of *M. robertsii*. These findings are evidence that active nitrogen acquisition by plants in this tripartite interaction may play a larger role in soil nitrogen cycling than previously thought.

Nitrogen gas, although it constitutes 78% of the atmosphere, is unavailable to plants as a source of nitrogen unless it is fixed by microbial symbionts (e.g., *Rhizobium*) or free-living microbes (e.g., *Azotobacter*) (1). In many natural as well as agricultural settings, nitrogen is the limiting nutrient for plant growth. The current model of the soil nitrogen cycle relies heavily on nitrogen-fixing bacteria to furnish plants with usable nitrogen (some is fixed by lightning strikes) (2). However, there are some examples in which plants have evolved mechanisms to scavenge nitrogen from insects. Carnivorous plants are able to obtain substantial amounts of nitrogen from insects they ingest. Pitcher plants (families

Nepenthaceae and Sarraceniaceae) trap insects in a deep cavity filled with liquid, and insect-derived nitrogen can constitute up to 70% of the plant nitrogen content (3). In one known case of fungus-mediated transfer of insect-derived nitrogen to plants, the ectomycorrhizal fungus *Laccaria bicolor* transfers nitrogen from soil-dwelling collembola to white pine (*Pinus strobus*) whose roots it colonizes (4).

The ability of *L. bicolor* to transfer insect-derived nitrogen was specific to white pine, and generally *L. bicolor* associates with roots of pine and spruce in temperate forests (5, 6). Nonetheless, these findings suggest that a more general example of insect-derived nitrogen transfer via fungal mycelia to plants may exist. *Metarhizium* spp. are ubiquitous soil-dwelling insect-pathogenic fungi that are found in a variety of ecosystems worldwide (7), occur in soils up to 10⁶ propagules per gram (8), and can infect more than 200 species of insects (9). Insects contribute substantial

amounts of nitrogen to soil. Each square meter of habitat can provide 0.4 to 4 g (by weight) of available insect nitrogen (see supplementary text).

During a routine survey of plant root symbionts, we found that *Metarhizium* spp. formed endophytic associations with many plant species (10, 11). Endophytes live internally within the plant, and the host plant may benefit from the interaction (12). Here, we hypothesized that *Metarhizium* can parasitize and kill a soil-born insect, then transfer the insect-derived nitrogen to plants via fungal mycelia and endophytic association.

We used ¹⁵N-labeled waxmoth (*Galleria mellonella*) larvae as a model prey insect and used this model in the experimental design to measure *Metarhizium*-mediated translocation of ¹⁵N to the foliage of haricot bean (*Phaseolus vulgaris*) or switchgrass (*Panicum virgatum*). ¹⁵N-labeled waxmoth larvae were added to microcosms in which the roots of the plants were separated from each insect by means of a 30-μm mesh (fig. S1). The insects were infected by *Metarhizium* 48 hours after ¹⁵N injection and then placed into the microcosm, and the amount of ¹⁵N transfer to plant tissues was determined during a 1-month period. After 14 days, in the presence of *Metarhizium*, insect-derived nitrogen constituted 28% and 32% of the nitrogen content in haricot bean and switchgrass, respectively; this represented significantly greater ¹⁵N incorporation than in the presence of uninfected ¹⁵N-labeled waxmoth larvae [Fig. 1, factorial analysis of variance (ANOVA), *P* < 0.01]. After 28 days, insect-derived nitrogen constituted 12% and 48% of bean and switchgrass nitrogen content, respectively, in the presence of *Metarhizium*; this again represented significantly greater ¹⁵N incorporation than in the presence of uninfected ¹⁵N-labeled waxmoth larvae (Fig. 1; factorial ANOVA, *P* < 0.05). Similar results were observed when the plant seeds were first inoculated with conidia of *Metarhizium* and subsequently formed a root endophytic association. We therefore concluded that

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Fig. 1. Percentage of plant nitrogen derived from waxmoth larvae by an endophytic, insect-pathogenic fungus. Two plant species were used: haricot bean (*Phaseolus vulgaris*) (A to C) and switchgrass (*Panicum virgatum*) (D to F). Shown are results obtained with *Metarhizium robertsii* [(A) and (D)], with *Aspergillus flavus* [(B) and (E)], and without fungus [(C) and (F)]. Nitrogen source: solid circles, waxmoth larvae; open circles, no waxmoth larvae. The amount of insect-derived nitrogen in the plant issues was determined with a NOI-5 emission spectrophotometer. Nitrogen content was calculated as $100 \times \%^{15}\text{N}$ in seedling leaves. Results were analyzed using *t* tests. Error bars represent 1 SE; *N* = 6. Results are the means of three separate trials done in duplicate.

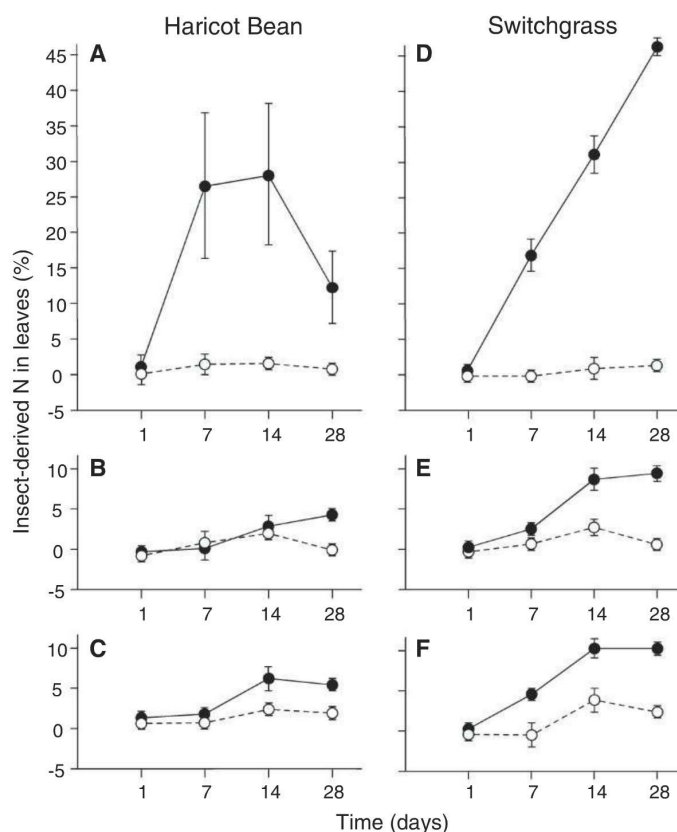
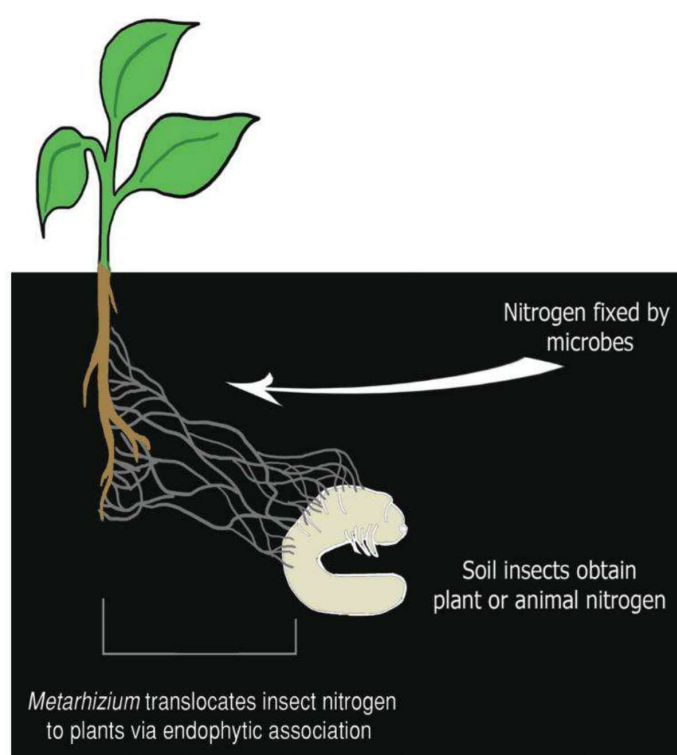


Fig. 2. Representation of the transfer of the insect-derived nitrogen to plants through an association with endophytic, insect-parasitic *Metarhizium*. *Metarhizium* infects and kills a soil-born insect. From the dead parasitized insect, fungal mycelia then associate endophytically with plant roots, through which nitrogen translocation occurs. The reverse may also occur; that is, endophytically associated *Metarhizium* could parasitize and kill an insect.



the hyphae infected the insect and transferred nitrogen back to the plant.

Plants grown in a soil microcosm containing a non-endophytic insect pathogen (*Aspergillus*

flavus strain 6982) (13) in the presence of ^{15}N -labeled waxmoth larvae contained less than 10% insect-derived nitrogen, which was not significantly different from plants grown in the presence of

uninfected ^{15}N -labeled waxmoth larvae (Fig. 1; factorial ANOVA, $P > 0.05$). In this experiment, insects were infected by *M. robertsii* or *A. flavus* within 2 days of placement into the microcosm. Insects were alive when placed into the microcosm but within two days of inoculation the larvae were dead. Six days after inoculation insects were mummified with fungal conidia, this was not observed in control experiments (fig. S2). Fungal propagules of both species were found within 0.5 cm of the plant roots within 6 days (fig. S3). Liquid chromatography–mass spectrometry was used to confirm the incorporation of insect-derived ^{15}N into plant amino acids (table S1).

Our results show that these plants derived a significant proportion of nitrogen from soil insects through their endophytic associations with the insect pathogen, *Metarhizium* spp. (Fig. 2). Possibly *M. robertsii* provides nitrogen to the plant in exchange for carbon. A plant carbon transporter, Mrt (*Metarhizium* raffinose transporter), has been reported for *Metarhizium* and is required for successful root colonization (14). Symbiotic association of *Metarhizium* with plants may aid in plant survival in nitrogen-limited soils as vectors for acquiring nitrogen from insects.

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Acknowledgments: Supported by a Natural Sciences and Engineering Research Council of Canada Discovery Grant (M.J.B.). We thank M. K. Bidochka for Fig. 2.

Supplementary Materials

www.sciencemag.org/cgi/content/full/336/6088/1576/DC1
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22 March 2012; accepted 2 May 2012
10.1126/science.1222289

The Dorsal Aorta Initiates a Molecular Cascade That Instructs Sympatho-Adrenal Specification

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The autonomic nervous system, which includes the sympathetic neurons and adrenal medulla, originates from the neural crest. Combining avian blood vessel–specific gene manipulation and mouse genetics, we addressed a long-standing question of how neural crest cells (NCCs) generate sympathetic and medullary lineages during embryogenesis. We found that the dorsal aorta acts as a morphogenetic signaling center that coordinates NCC migration and cell lineage segregation. Bone morphogenetic proteins (BMPs) produced by the dorsal aorta are critical for the production of the chemokine stromal cell–derived factor–1 (SDF 1) and Neuregulin 1 in the para-aortic region, which act as chemoattractants for early migration. Later, BMP signaling is directly involved in the sympatho-medullary segregation. This study provides insights into the complex developmental signaling cascade that instructs one of the earliest events of neurovascular interactions guiding embryonic development.

The autonomic nervous system (ANS) plays a pivotal role in a wide range of physiological functions, including homeostasis and stress defense, and dysfunction of the ANS leads to a variety of diseases (1). However, it remains largely unexplored how the ANS is established during development at the cellular and molecular levels.

The ANS consists of sympathetic and parasympathetic neurons, as well as stress defense tissues, including the adrenal gland. In the trunk of the early developing embryo, progenitors of sympathetic ganglion (Sg) emerge as a derivative of neural crest cells (NCCs), an embryonic progenitor cell population emigrating from the dorsal aspect of the neural tube (2). In a defined region along the anteroposterior (AP) body axis [somite pairs 18 to 24 in chicken embryos (3, 4)], Sg-progenitor cells also give rise to adrenal medulla (Am) cells, which later become associated with the adrenal cortex, a tissue of different origin. Thus, Sg cells and Am cells are produced from common progenitors (SA progenitors). The SA progenitors first migrate ventrally toward the dorsal aorta, the first-formed embryonic blood vessel, and subsequently, these cells segregate into the Sg and Am lineages in the para-aortic region (5–8). Thus, the dorsal aorta has long been anticipated to play a role in the regulation of sympatho-adrenal lineages (9). However, its mechanism of action remains elusive, mainly because it has been difficult to manipulate the dorsal aorta (blood vessel) and sympatho-adrenal lineages

simultaneously and separately in the same embryo. We demonstrate here that the dorsal aorta acts as a morphogenetic center for SA migra-

tion and Sg-Am segregation, and bone morphogenetic proteins (BMPs) play critical roles for these activities.

Three cytokine families, BMPs, stromal cell–derived factor–1 (SDF1, a chemokine, also called CXCL12), and Neuregulin 1 [NRG1 of the epidermal growth factor (EGF) family] have been separately implicated in the generation of SA progenitors from naïve NCCs (6, 10–12). We therefore asked if and how these factors were related to the function of the dorsal aorta. We found that BMP signals were widely received by the cells located in a para-aortic area. These cells included SA progenitors [NCC marker antibody HNK-1–positive] (Fig. 1A) (13), whose response to BMPs was revealed by their staining with antibody against phosphorylated Smad1, 5, and 8 (pSmad) (14, 15). The BMP ligands (BMP4 and BMP7), produced by the dorsal aorta (fig. S1), were critical for the SA progenitors to be properly positioned, as transfection with the BMP inhibitor *Noggin* cDNA specifically into the dorsal aorta extinguished the para-aortic pSmad signal and abrogated SA progenitors completely (Fig. 1, B to D, and G to I). This notion was further supported by unilateral overexpression of

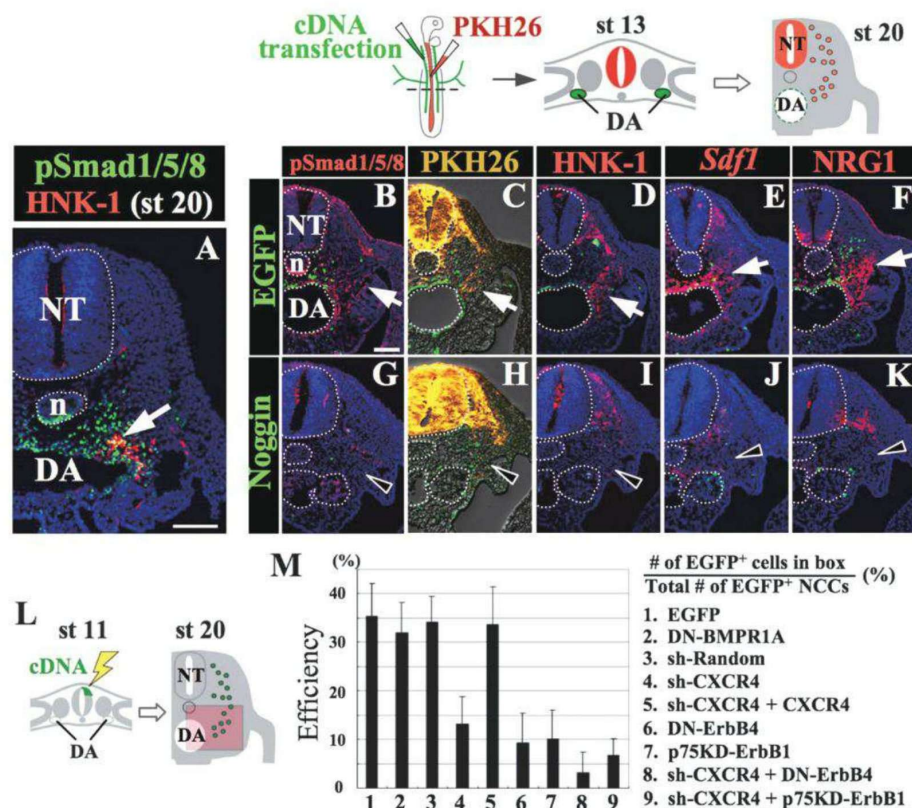


Fig. 1. SDF1 and NRG1, but not BMPs, act directly on migrating SA progenitors. (A) NC-derived sympathetic precursors (HNK-1 positive) were active for BMP signals (pSmad1, 5, and 8 positive). Diagram: DNA-transfection into the dorsal aorta and red fluorochrome (PKH26)–labeling of NCCs of a single embryo. Transfection with EGFP (B to F) or Noggin (G to K). Arrows (EGFP control) and arrowheads (Noggin-transfected) indicate corresponding regions. (L) Electroporation into NC. (M) Quantitative representation of the ratio of electroporated NCCs located in the para-aortic region (square) over the total number of electroporated NCCs. The data were obtained by those shown in fig. S3. Scale bars, 100 μ m. NT, neural tube; n, notochord; DA, dorsal aorta.

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Noggin, which resulted in a unilateral abrogation of SA progenitors (fig. S2) (16). However, the effects of BMPs on the SA accumulation were not direct, because SA progenitors electroporated with a dominant-negative type of BMP receptor (DN-BMPRI1A) (17) migrated almost normally toward the aorta (Fig. 1, L and M, and fig. S3), consistent with a previously reported mouse genetic study (18).

We therefore investigated the roles of SDF1 and NRG1, previously shown to be distributed in the para-aortic mesenchyme (10, 11) (fig. S4). Our data strongly suggest that these cytokines are the direct mediators for SA-progenitor migration and/or distribution. Functional inhibition of their respective receptors, CXCR4 (fig. S4) or EGF family ErbBs, in NCCs by short hairpin RNA (19) or DN-type constructs (DN-ErbB4, p75KD-ErbB1) (20, 21) profoundly disturbed the SA cell accumulation in the para-aortic region (Fig. 1, L and M, and fig. S3). Simultaneous knockdown of these two receptors enhanced the phenotype (fig. S3). Furthermore, the para-aortic expression of *SDF1* and *NRG1* was extinguished either bilaterally or unilaterally by *Noggin* overexpression in the dorsal aorta or a unilateral site, respectively (Fig. 1, E, F, and J to K, and fig. S2). Thus, the aortic BMPs are required for the expression of SDF1 and NRG1 in the para-aortic mesenchyme, which in turn directly regulate SA cell morphogenesis.

Both in vivo and in vitro analyses further revealed that SDF1 and NRG1 act as chemoattractants for the SA progenitors. When an aggregate of NRG1- or SDF1-producing DF1 cells (chicken mesenchymal cell line) was implanted into a

somitic area, an ectopic accumulation of neural crest (NC)-derived cells was observed near the implant (Fig. 2, A to F, and fig. S5, A and B). Such ectopic accumulation also occurred when a piece of dorsal aorta was transplanted into a similar region (fig. S6, A to C). In these cases, expression of *SDF1* and *NRG1* was up-regulated in mesenchymal cells near the graft (fig. S6, D and E). Implantation of BMP-producing DF1 cells resulted in neither up-regulation of *SDF1* and *NRG1* nor NCC accumulation (fig. S5, C and D). Together, we conclude that the dorsal aorta initiates the expression of SDF1 and NRG1, which in turn attract the SA progenitors. For these events, the aortic BMPs are required.

The direct attraction by SDF1 and NRG1 was further verified by in vitro time-lapse imaging analyses. A clump of NCCs prepared from early embryos was cocultured in Matrigel with an aggregate of SDF1- or NRG1-producing DF1 cells (Fig. 2G). The NCCs exhibited directional migration toward SDF1- or NRG1-producing cells (movies S2 and S3), but not to enhanced green fluorescent protein (EGFP)-positive or BMP4-producing cells (movies S2 and S4), although the NCCs maintained motility (Fig. 2H and fig. S7). Thus, SDF1 and NRG1, but not BMPs, act as chemoattractants on migrating SA progenitors. This work identifies how the dorsal aorta directs the migration of SA progenitors, showing that this morphogenetic event involves multiple temporally and spatially regulated factors.

We further explored the mechanisms underlying the late-occurring event, Sg-Am segregation from SA progenitors. To our surprise, upon reaching the aortic region, SA progenitors turned

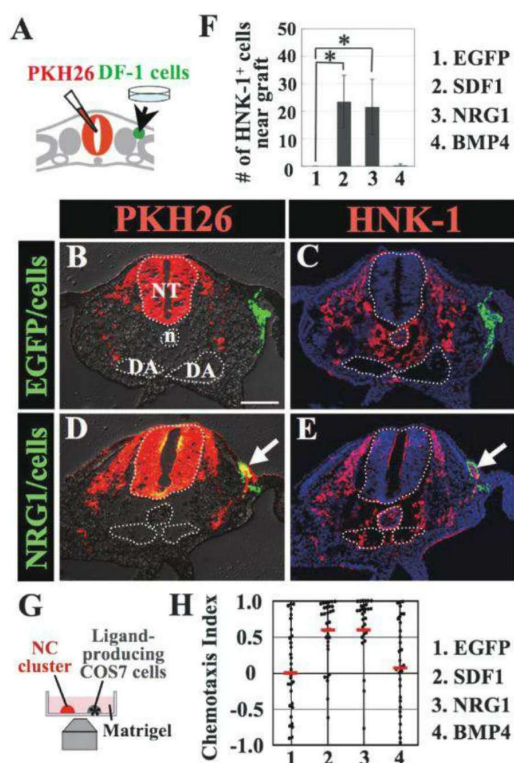
off the pSmad signal at embryonic stage (st) 21 (Fig. 3, A and B). Subsequently, the pSmad signal reappeared at st 23, but, this time, the pSmad labeling was seen only in the Am cells and not in the Sg cells (Fig. 3C). To determine whether this asymmetric distribution of BMP signaling actually would cause the Am-Sg segregation, we inhibited BMP signals specifically in these Am-Sg cells through tetracycline (tet)-controlled conditional expression (22, 23). Thus, the expression of tet-DN-BMPRI1A was turned on at st 20 just before Am-Sg segregation (fig. S8A). Whereas control EGFP-electroporated cells were randomly distributed both in Sg and Am populations, DN-BMPRI1A-expressing cells failed to participate in Am production (Fig. 3, D to F), which suggested that the BMP signaling is essential for the Am-Sg segregation. BMP ligands, *BMP4* and *BMP7*, are most likely provided by the dorsal aorta (fig. S1, D and E). In support of this, tet-controlled *Noggin* expression in the dorsal aorta at st 20 disturbed Am cells, but not Sg cells, seen in both sides of the aorta (Fig. 3, G and H, and fig. S8, D to H). Unilateral transfection further confirmed this notion (fig. S8, I to L).

NRG1 was also important for the Am cells. NRG1 was localized along the migration pathway of Am cells, so that these cells were enclosed by NRG1-positive areas (Fig. 3K). NRG1-ErbBs signaling was necessary for proper Am-cell segregation, as SA progenitors expressing tet-DN-ErbB4 or tet-p75KD-ErbB1 failed to differentiate into Am cells (Fig. 3F and fig. S8, B and C).

To determine whether BMPs and NRG1 play distinct roles in Am morphogenesis, we placed a BMP4- or NRG1-producing cell aggregate into an ectopic site ventral to the aorta (fig. S8M) and found that the NRG1-aggregate, but not the BMP4, could attract Am cells to the ectopic site (Fig. 3, L and M, and fig. S8N). Thus, NRG1 and BMP4 exert different effects. It is noteworthy that BMPs are required for the NRG1 distribution, as tet-controlled *Noggin* cDNA overexpressed in the aorta extinguished the NRG1 signals along the migration pathway of Am cells (Fig. 3, I and J). Together, BMP4 and 7 signaling are required for both the Am segregation and NRG1 patterning, whereas NRG1, in turn, acts as an attractant for Am-cell displacement.

Most previous studies of Am morphogenesis focused on possible interactions between Am cells and adrenal cortex, because these two tissues become associated to constitute the adrenal gland (24–26). However, even in the cortex-deficient mice produced by knocking out the gene for transcriptional factor steroid factor 1 (*Sf1* or *Ad4BP*) (27, 28), about half the Am cells are present (29), which leaves the role of cortical cells undefined. Based on our observations (Fig. 3K), we reasoned that even when deprived of the cortex, NRG1 expression would remain in other areas and provide guidance cues for the Am cells. Indeed, in addition to the NRG1-positive cortex (*Sf1*^{+/+}/*BMP4*^{+/+}) (30), para-aortic mesenchyme was found to express NRG1 (Fig. 4, A and B).

Fig. 2. SDF1 and NRG1 on SA progenitors act as chemoattractants. (A) SDF1- or NRG1-producing DF1 cells (EGFP-positive) were implanted into a somitic area of a st 13 embryo. (B to E) PKH26, HNK-1, and EGFP signals were visualized in the same histological section of st 23 embryos. Arrows, Ectopic accumulation of NCCs. Scale bar, 100 μ m. (F) The number of NCCs found within a 20- μ m distance from the implant, assessed with 50 histological sections obtained from 10 embryos for each manipulation ($*P < 0.001$). Error bars represent SEM. (G and H) In vitro live imaging and chemoattraction analyses of NCCs. Migratory tracks of individual NCCs exposed to a given factor, as shown in fig. S7, are represented by Chemotaxis Index (see supplementary material). Dots represent index of individual cells ($*P < 0.05$). Red bar, average index.



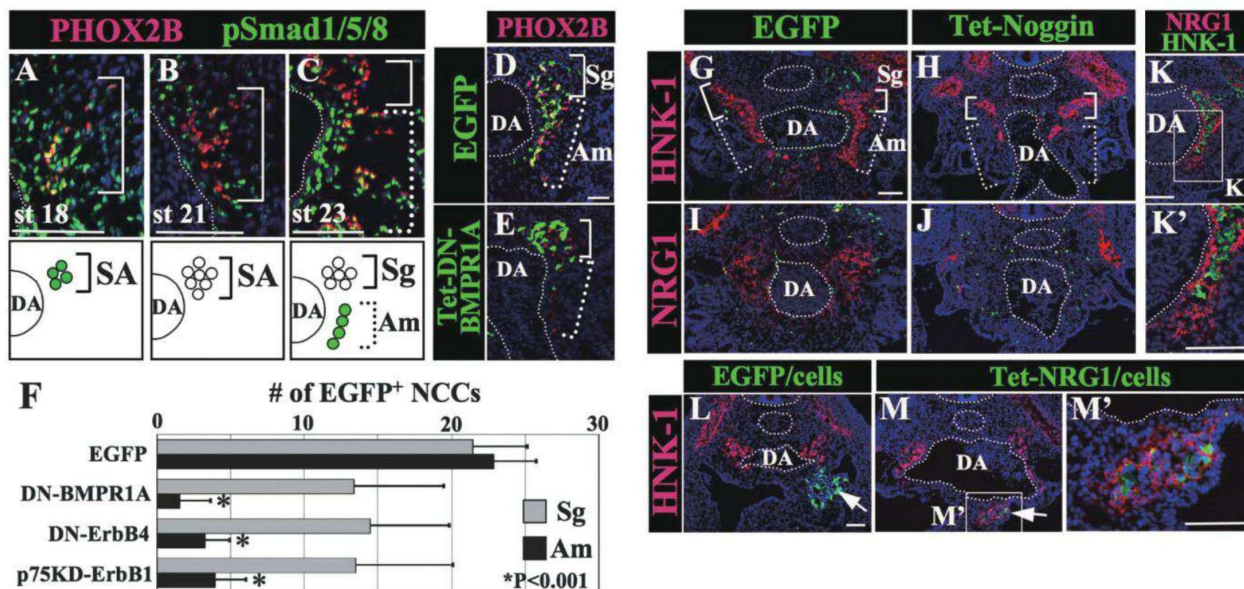
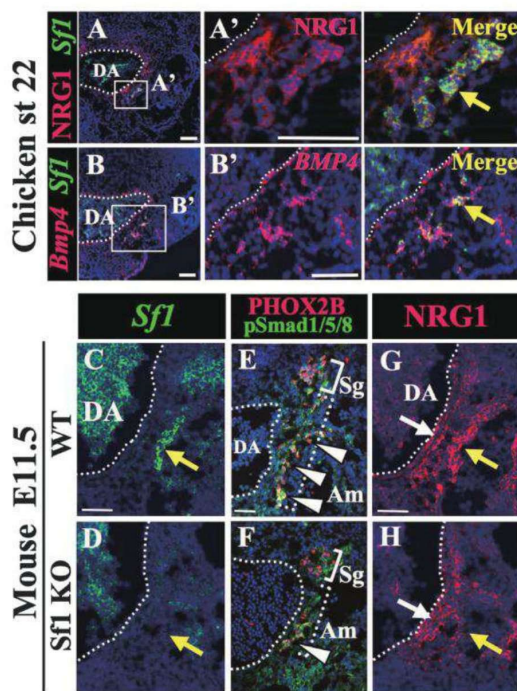


Fig. 3. BMPs and NRG1 determine the late event of Am-Sg segregation. (A to C) Double staining for NCCs (PHOX2B) and BMP signals. Solid and broken brackets indicate SA/Sg cells and Am cells, respectively. (D and E) Tet-induced expression of DN-BMPR1A into SA lineage (see fig. S8A for experimental design) abrogated Am cells. (F) The number of electroporated cells (EGFP+) located in Sg and Am populations. Error bars, SEM. (G to J)

Tet-induced expression of *EGFP* or *Noggin* cDNAs transfected into the dorsal aorta (see fig. S8D). (K) NRG1 localization near Am cells at st 23. (L and M) DF1 cells, transfected with *tet*-inducible *EGFP* or *NRG1* genes (green), were implanted into coelomic mesoderm at st 11 (arrows) (see fig. S8M for experimental design). (K') and (M') are magnified fields. Scale bars, 100 μ m.

Fig. 4. Am cells are guided by two distinct NRG1-positive domains. (A and B) Transverse views of Am cells near the dorsal aorta of chicken embryos. (A' and B') Magnified fields. Yellow arrows indicate cortex precursors (*Sf1*⁺/*BMP4*⁺). (C to H) Para-aortic regions in mouse wild type- and *Sf1* KO embryos (E11.5). Yellow arrows show the areas that correspond to adrenal cortical precursors. White arrowheads and white arrows indicate Am cells and NRG-positive para-aortic mesenchyme, respectively. Scale bars, 100 μ m.



To determine whether the NRG1 signal would remain near the Am cells when deprived of the cortex, we examined the para-aortic region of *Sf1* knockout (KO) mice (Fig. 4, C and D) (28). The distribution patterns of pSmad proteins and NRG1 in wild-type mouse embryos at the time of Am-cell segregation from SA progenitors, embryonic day 11.5 (E11.5), were virtually the same

as in the chicken embryo (Fig. 4, C, E, and G). In *Sf1* KO mice at E11.5, a substantial number, although smaller than wild type, of Am cells were detected as previously reported (29). These cells exhibited pSmad1, 5, and 8 (Fig. 4F, white arrowhead). Furthermore, NRG1 was maintained near the Am cells in KO mice; the signal intensity in the para-aortic mesenchymal area (Fig. 4, G

and H, white arrow) was comparable between KO and wild type, whereas the area that would normally be occupied by the cortex exhibited little signal in KO mice (Fig. 4, G and H, yellow arrow). These findings thereby explain how Am cells could survive in *Sf1* KO mice: The Am cells can receive aortic BMP signals that allow them to be segregated from Sg cells, and these Am cells are guided by the NRG1 that remains in the para-aortic region (fig. S9).

In this study, we have unveiled the molecular and cellular mechanisms by which the sympathetic nervous system and stress defense organ are established (fig. S9). This occurs in the vicinity of the dorsal aorta, where multiple factors are repeatedly used in a manner regulated spatiotemporally by the dorsal aorta. The dorsal aorta controls the migration of early SA progenitors, Sg-Am segregation, and the ventral displacement of Am lineage. For these sequential events to proceed correctly, cells constantly change their responsiveness to factors they encounter (i.e., their BMP responsiveness). The dorsal aorta thus serves as a critical relay point to coordinate the diversification of sympathetic nervous system.

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Acknowledgments: We thank S. F. Gilbert, L. Niswander, G. Corfas, S. O. Yoon, and Y. Wakamatsu for experimental materials and discussions and K. Morohashi for providing us with *Sf1* KO mice. This work was supported by a Grant-in-Aid for Scientific Research on Innovative Areas, and Grant-in-Aid for Scientific Research (A) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (MEXT); the Global Centers of Excellence (GCOE) program (Frontier Biosciences, Nara Institute of Science and Technology) from MEXT, Japan; CREST (JST); The Mitsubishi Foundation; and Takeda Science Foundation. Y. Takase is a fellow of the Japan Society for the Promotion of Science.

Supplementary Materials

www.sciencemag.org/cgi/content/full/336/6088/1578/DC1
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22 November 2011; accepted 8 May 2012
10.1126/science.1222369

Membrane Fusion Intermediates via Directional and Full Assembly of the SNARE Complex

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Cellular membrane fusion is thought to proceed through intermediates including docking of apposed lipid bilayers, merging of proximal leaflets to form a hemifusion diaphragm, and fusion pore opening. A membrane-bridging four-helix complex of soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) mediates fusion. However, how assembly of the SNARE complex generates docking and other fusion intermediates is unknown. Using a cell-free reaction, we identified intermediates visually and then arrested the SNARE fusion machinery when fusion was about to begin. Partial and directional assembly of SNAREs tightly docked bilayers, but efficient fusion and an extended form of hemifusion required assembly beyond the core complex to the membrane-connecting linkers. We propose that straining of lipids at the edges of an extended docking zone initiates fusion.

Synaptic vesicle fusion is mediated by the vesicular *N*-ethylmaleimide-sensitive (NSF) factor attachment protein receptor (SNARE)

synaptobrevin 2 (syb) and the plasma membrane SNARE's soluble NSF attachment protein SNAP-25A (SN25) and syntaxin 1A (syx). Binding of syb to a 1:1 syx:SN25 acceptor complex forms a SNARE complex consisting of a four-helix bundle held together by 16 layers (numbered –7 to +8) of interacting amino acids (*1*). The SNARE complex alone is sufficient for mediating fusion in vitro (*2*); the prevalent model is that directional N- to C-terminal zippering of a trans SNARE complex pulls opposing membranes together. Once contact has been established, fusion is thought to proceed via a symmetrical stalk intermediate that expands to form a hemifusion diaphragm (*3*) (Fig. 1A). An expanding pore formed within the diaphragm then completes fusion. Some studies have reported the involvement of some of these intermediates (*4*); others have attempted to arrest them by disrupting SNARE assembly (*5*); however, key questions remain unanswered, including what is the nature of the relevant dock-

ing interaction that leads to fusion, and—just as important—how does the assembly of the SNARE complex give rise to docking and other intermediates that occur thereafter?

To address these questions, we pursued a minimalist approach in which docking and fusion is recapitulated with purified SNAREs reconstituted in liposomes. Previously, a transient docking state was inferred when liposomes were used that contained a syx:SN25 acceptor complex stabilized by a C-terminal syb fragment (Δ N syb 49–96, hereafter denoted Δ N syb) (Fig. 1B) (*6*). This “ Δ N complex” contains a free binding site for syb at the N terminus, which accelerates syb trans binding and prevents dead end 2:1 syx:SN25 complexes (*7*). Docking of large SNARE-liposomes (radii ~40 to 100 nm) (Fig. 1C) had a longer lifetime than smaller ones (radii ~15 to 25 nm), a finding we attribute to reduced curvature stress (*8, 9*) of the large liposomes, which approaches that of giant liposomes (Fig. 1C inset). We confirmed this behavior in fluorescence resonance energy transfer (FRET)-based lipid mixing assays. Here, a pronounced lag phase was observed with large liposomes, which suggested that lower curvature delays the initiation of fusion once membrane contact has been established (Fig. 1D).

To monitor trans SNARE-complex binding and Δ N syb displacement, the experiments were repeated with fluorophore-labeled versions of full-length syb (syb^{28A488}) and Δ N syb (Δ N syb 49–96^{79A488}). Accordingly, SNARE-complex zippering began without delay at the very N terminus (Fig. 1E), but Δ N syb displacement slowed down further zippering toward the C-terminal end (Fig. S2). However, this delay originated only from the displacement of Δ N syb's first N-terminal layers, whereas the remaining C-terminal half of Δ N syb dissociated rapidly thereafter, which allowed zippering to proceed (Fig. S3). On the basis of these findings, we reasoned that the delay between trans SNARE binding and the initiation of lipid mixing, caused first by Δ N syb displacement and

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then more prominently by the low membrane-curvature stress of the large liposomes used here (Fig. 1D), opens a time window to identify intermediate states of the membranes on their way to fusion by electron microscopy (EM).

We found many docked liposomes during the lag phase by negative stain (Fig. 2A) and cryoEM (Fig. 2, B to D) and distinguished docked membranes with both minimal (Fig. 2B) and extended docking zones (Fig. 2, C and D). Because none of these interactions implicate any lipid mixing and occur during the lipid-mixing lag phase where SNARE assembly takes place, we hypothesized that these docked states are generated by partial complex zippering on their way to fusion. To our surprise, we also identified a small number of liposomes containing only one bilayer in between the two lumina (Fig. 2E), the ultrastructural signature of an extended hemifusion diaphragm (Fig. 2F). Hemifusion (like docking) was SNARE-dependent, an important distinction given that even misfolded proteins can mediate hemifusion (10) (fig. S4). However, we were unable to distinguish small differences in the counting of docking and hemifusion by EM, which prevented us from making definite conclusions about the reaction sequence.

To clarify this question, we resorted to alternative biophysical assays and used a combination of fluorescence cross-correlation spectroscopy (FCCS) and FRET to follow the evolution of docking (6). FCCS-FRET revealed an initial and instantaneous accumulation of docking (without fusion) followed by a plateau (Fig. 2G), which suggested that all docked states observed by EM are transient intermediates and are part of

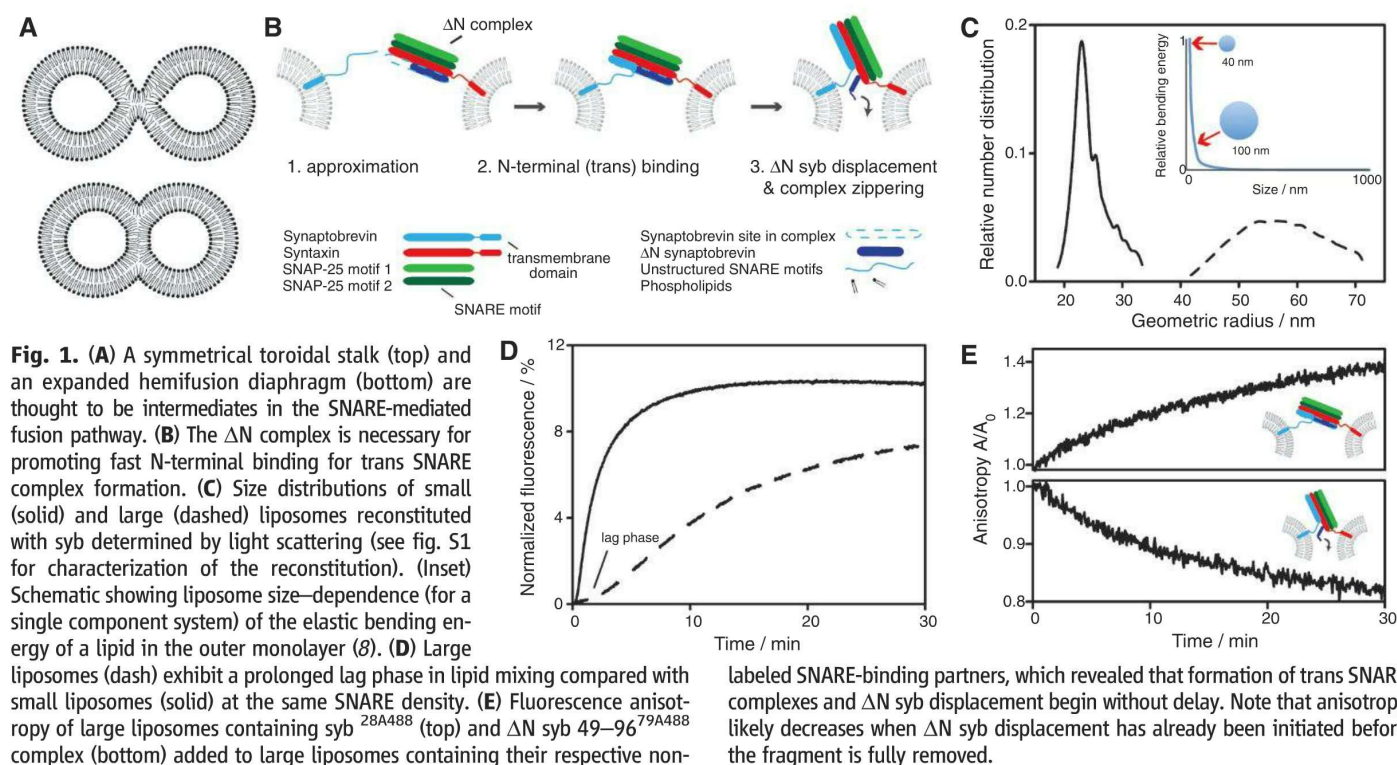
the same pathway. This finding appeared initially to contradict the observation that many docked liposomes were still seen by EM after lipid mixing was completed (fig. S4), but we note that FCCS-FRET does not detect docking between liposomes that have already docked and fused previously. We therefore postulate that docked liposomes at the end of the reaction have already fused at least once but contain insufficient SNAREs for additional cycles of fusion. Although one SNARE complex is sufficient for fusing small liposomes (11), it appears that more complexes are required for fusing larger ones (see below).

To monitor the evolution of hemifusion, we measured lipid mixing in the inner leaflet of a merging bilayer (12, 13) and compared it with the expected value when full-fusion (i.e., no hemifusion) conditions are assumed (Fig. 2H and fig. S5). Inner-leaflet lipid mixing begins to diverge after ~3 to 5 min from the expected value (Fig. 2H), which means that hemifusion begins to develop mainly after the lag phase and that it arises sequentially from docked intermediates identified by EM. Note that inner-leaflet lipid mixing remained below the expected value throughout the rest of the reaction, which indicated that hemifusion accumulates and becomes kinetically trapped, in agreement with the appearance of hemifused liposomes with extended diaphragms seen at the end of the reaction by EM (fig. S4).

To pinpoint the regions of the SNARE complex responsible for tight docking, extended hemifusion, and fusion, we sought to arrest intermediates by perturbing SNARE assembly at

the transition between the four-helix bundle and the membrane-connecting linker, a region with a critical role in fusion (5, 14, 15). To this end, we made a single deletion at position 84 of syb (syb $\Delta 84$) that disrupts the last +8 layer of the bundle. Syb $\Delta 84$ was unable to fuse large liposomes, although it partially induced lipid mixing on small ones, a result consistent with our earlier supposition that large liposomes require more energy (and, therefore, more SNARE complexes) to fuse (Fig. 3A). We confirmed that syb $\Delta 84$ displaced the ΔN syb fragment from the ΔN complex (fig. S6), which suggested that it could assemble into SNARE complexes and dock liposomes but not fuse them. This was confirmed by FCCS-FRET (Fig. 3B) and EM, where almost all liposomes were seen arrested at the tightly docked state with extremely rare sightings of hemifusion (Fig. 3, C and D).

Several key conclusions can be derived from this analysis: (i) tight docking is a result of partial complex assembly which does not exceed layer +7, which validates our hypothesis that tightly docked liposomes are a result of partial zippering; (ii) assembly beyond the +7 layer of the SNARE complex is essential for generating extended hemifusion and fusion; (iii) in line with directional N- to C-terminal assembly (16), the tight bilayer arrangement with extended contact zones represents an intermediate state that was stalled on its way to fusion. Further supporting this conclusion, lipid mixing by syb $\Delta 84$ can be partially restored by increasing curvature, which suggests that large liposomes would otherwise fuse were it not for its higher energy barrier (Fig. 3A); (iv) because bilayers



labeled SNARE-binding partners, which revealed that formation of trans SNARE complexes and ΔN syb displacement begin without delay. Note that anisotropy likely decreases when ΔN syb displacement has already been initiated before the fragment is fully removed.

are tightly held together with no resolvable space in between, complexes are arrested in trans and are probably distantly distributed along the vertex ring as described in yeast vacuoles (17); and (v) lipids at the edges of the extended docking zone are highly strained where splaying of lipid tails or stalks are likely to initiate fusion (3, 18).

We took advantage of this “docking mutant” and tested if the Ca^{2+} sensor synaptotagmin (syb),

which might stabilize fusion intermediates by bending bilayers (19), can assist SNAREs in the conversion from docking to hemifusion or fusion but found it could restore neither, consistent with the view that syt operates upstream of complex assembly (20) (fig. S7). We therefore turned to our mutational analysis by exploring single and double deletions further downstream of syb in the linker region (Fig. 4C, inset), ensuring complete assembly of the core four-

helix bundle (see fig. S8 for characterization of syb linker mutants). Both fusion and extended hemifusion were restored for all three mutants tested, albeit with lower efficiency than wild-type syb (Fig. 4A and fig. S8C), which confirmed that hemifusion is preceded by tight docking and showed that efficient fusion requires zippering of the linker in agreement with the crystal structure of the full SNARE complex (21). In addition, the hemifusion/fusion ratio was

Fig. 2. Ultrastructural and biophysical identification of docking and hemifusion. **(A)** Negative-stain EM depicting liposomes engaged in docking taken at ~3 min after mixing. Scale bar, 200 nm. Docked liposomes with minimal **(B)** and extensive **(C and D)** contact zones were observed by cryoEM ~1 to 2 min after mixing. **(E and F)** Hemifused liposomes were identified by an extended diaphragm consisting of a single bilayer (arrow). Scale bar, 50 nm, except in **(F)**, where the scale bar is 20 nm. **(G)** Discrimination of docking and fusion by FCCS-FRET. Cross-correlations between labeled liposomes (reflecting both fused and docked liposomes, red) were subtracted from changes in fluorescence lifetime (reflecting fused liposomes, black) to reveal the evolution of docked liposomes (blue). Data are means $\pm$ SD ($N \geq 5$). **(H)** Total (black) and inner-leaflet (red) lipid mixing measured and compared with the expected inner-leaflet lipid mixing (cross-shaded region). Hemifusion begins to form after ~3 to 4 min and accumulates thereafter. Bars represent 95% confidence intervals ($N \geq 3$).

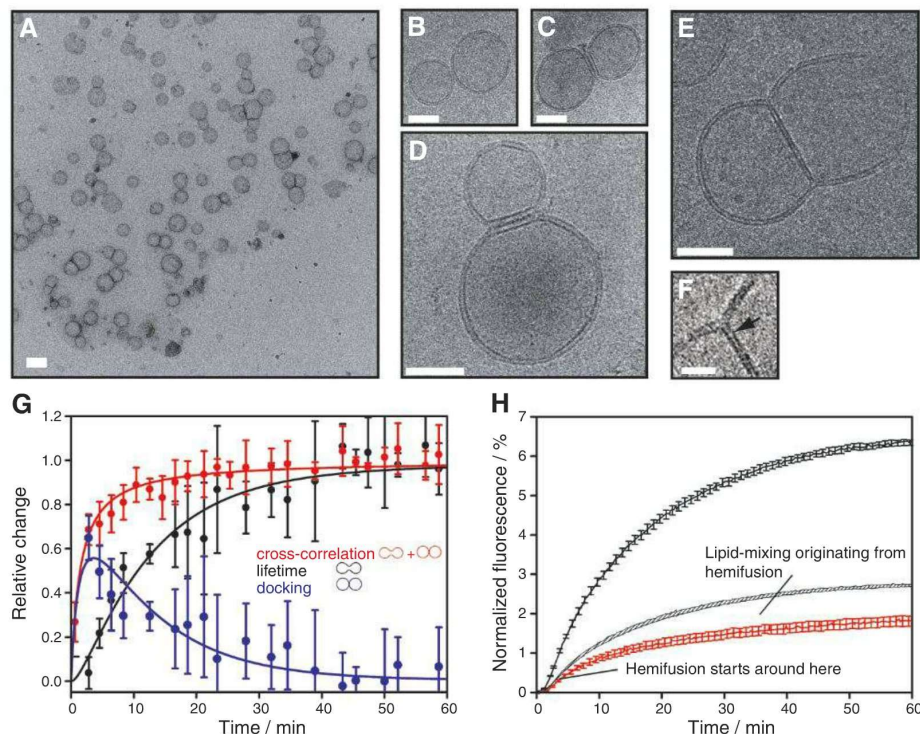
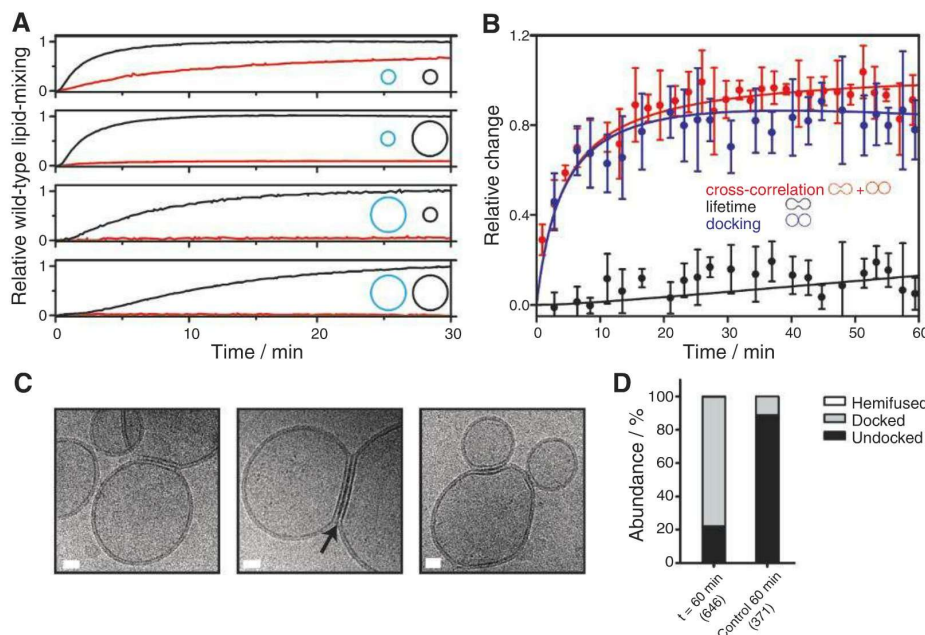


Fig. 3. Disruption of the C-terminal +8 layer of the SNARE complex arrests a tightly docked intermediate. **(A)** Comparative lipid mixing of wild-type syb (black) and syb $\Delta 84$ (red) according to the depicted liposome size combinations (location of ΔN complex is indicated in light blue), which shows that curvature affects the ability of syb $\Delta 84$ to mediate fusion. Traces were normalized to wild-type syb, which was set arbitrarily to 1. **(B)** FCCS-FRET analysis of large syb $\Delta 84$ liposomes showing accumulation of docking. Data are means $\pm$ SD ($N \geq 5$). **(C)** Examples of cryo-EM images of syb $\Delta 84$ and ΔN complex large liposomes depicting the arrest at the tightly docked state. The edges of an extended docking zone result in straining of lipids (arrow). Scale bar, 20 nm. **(D)** Counting of docking of syb $\Delta 84$ and ΔN complex liposomes observed by cryoEM after 1 hour, confirming the vast majority of liposomes were arrested in the tightly docked state. Control was performed in the presence of excess soluble syb 1–96 showing that docking was SNARE-dependent.



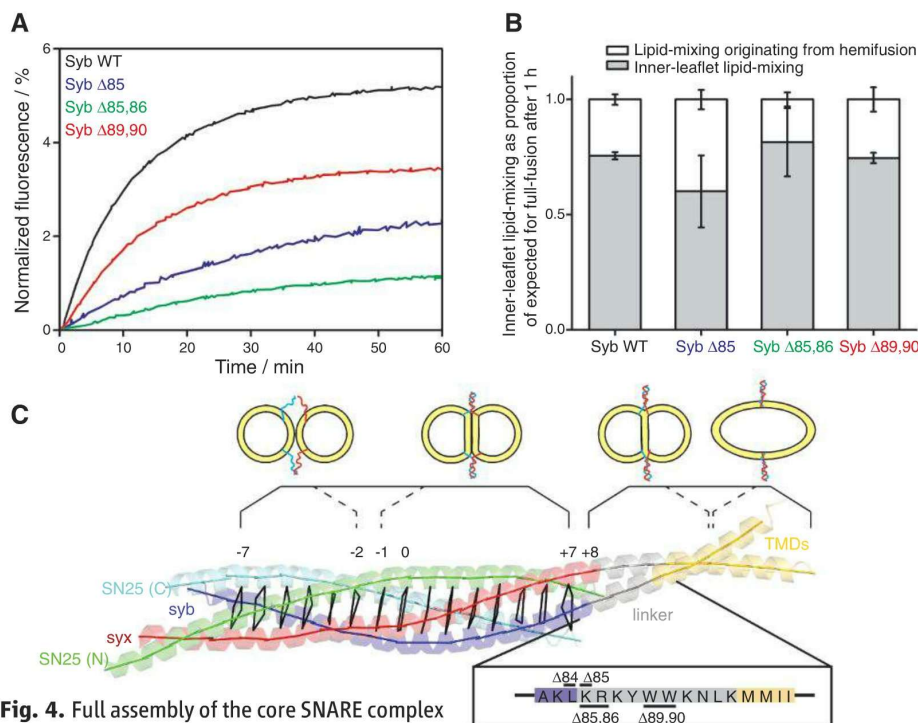


Fig. 4. Full assembly of the core SNARE complex restores hemifusion, and the linkers are needed for efficiently completing fusion. **(A)** Comparative lipid mixing of linker deletion mutants depicting a decrease in fusion in large liposomes. **(B)** Inner-leaflet lipid mixing as a proportion of expected, if one assumes full-fusion conditions for wild-type syb and linker deletion mutants. Values were taken 1 hour after mixing, and bars represent 95% confidence intervals ($N \geq 3$). **(C)** Ribbon structure (21) of the fully assembled SNARE complex showing the interacting layers (black lines, +8 layer indicated) of the four-helix bundle, the linker, and TMD and the proposed regions which give rise to docking, extended hemifusion, and fusion. Dashed lines indicate borders of regions that are based on other studies (22, 23) or on biochemical characterization. (Inset) Deleted amino acids used for mutation analysis.

unaffected (Fig. 4B), which indicated that the linkers were not involved in the transition from hemifusion to fusion, although this may be the role of the transmembrane domain (TMD) (22, 23).

On the basis of our findings, we can assign approximate regions responsible for the generation of docking, extended hemifusion, and fusion by directional and full assembly of the SNARE complex (Fig. 4C). Our conclusion that membrane merging begins after assembly of the core complex contrasts to a recent study that used myricetin to stall a partially zippered complex and arrest a topologically undefined hemifusion state (24). However, myricetin also binds to acyl chains (25) and may increase membrane fusogenicity or leakiness. Our finding that extended hemifusion accumulates suggests that it is a kinetically trapped intermediate resulting from a high-energy barrier for pore opening, a state that has been observed in cortical granules (26). Nevertheless, additional factors—for instance, specific lipid requirements—may lower that barrier and make the extended hemifusion conformation a viable intermediate, as suggested by studies of giant liposomes and simulations (27, 28), although it is still not clear whether a localized,

rather than an extended, form of hemifusion may also be a biologically relevant intermediate (see fig. S9 for a discussion).

Our findings question the routinely made assumption that fusion must start from a symmetrical toroidal stalk. Instead, the SNARE fusion machinery seems to work by pulling the membranes as tightly as possible, which strains the edges of an extended docking zone. Such a mechanism shares some similarities with what appears to occur in vacuoles (17) and was observed with greater detail in simulations (27, 29, 30). Our work now suggests that the tight pulling mechanism is a conserved feature of SNARE-mediated fusion.

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Acknowledgments: We thank G. van den Bogaart, J. Risselada, J. Chua, R. Klemm, and T. Rapoport for critical review; G. Weber for providing the ribbon structure; and O. Hofnagel and U. Ries for valuable technical assistance. Funding was generously provided by the National Institutes of Health (3P01GM072694-05S1 to R.J.), the German Research Foundation (RA 1781/1-1 to S.R. and SFB 803 to R.J. and P.J.W.), the “Fonds der Chemischen Industrie” grant 684052 to E.B. and grants 166660 and 661218 to P.J.W., and a doctoral fellowship from the National Commission for Scientific and Technological Research of Chile to J.M.H. Additional data described in the manuscript are presented in the supplementary materials.

Supplementary Materials

www.sciencemag.org/cgi/content/full/science.1221976/DC1
Materials and Methods
Supplementary Text
Figs. S1 to S9
References (31–53)

15 March 2012; accepted 11 May 2012
Published online 31 May 2012;
10.1126/science.1221976

The Fission Yeast FANCM Ortholog Directs Non-Crossover Recombination During Meiosis

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The formation of healthy gametes depends on programmed DNA double-strand breaks (DSBs), which are each repaired as a crossover (CO) or non-crossover (NCO) from a homologous template. Although most of these DSBs are repaired without giving COs, little is known about the genetic requirements of NCO-specific recombination. We show that Fml1, the Fanconi anemia complementation group M (FANCM)—ortholog of *Schizosaccharomyces pombe*, directs the formation of NCOs during meiosis in competition with the Mus81-dependent pro-CO pathway. We also define the Rad51/Dmc1—mediator Swi5-Sfr1 as a major determinant in biasing the recombination process in favor of Mus81, to ensure the appropriate amount of COs to guide meiotic chromosome segregation. The conservation of these proteins from yeast to humans suggests that this interplay may be a general feature of meiotic recombination.

Faithful chromosome segregation during meiosis depends on the establishment of chiasmata through recombinational repair of programmed DNA double-strand breaks (DSBs) to produce crossovers (COs) between homologous chromosomes (homologs). However, in most

cases only a minority of the DSBs are earmarked to form COs, and therefore the majority have to be repaired by using either the homolog without CO formation or the sister chromatid (I).

In order to identify helicase activities involved in non-crossover (NCO)—recombination during meiosis in the fission yeast *Schizosaccharomyces pombe*, we screened for helicases potentially capable of D loop unwinding during synthesis-dependent strand annealing (SDSA), which is thought to be a major pathway of NCO recom-

ination (I). To this end, we used a genetic recombination assay consisting of a meiotic recombination hotspot at the *ade6* gene and two flanking scorable markers (Fig. 1A). We hypothesized that at least one of the helicases promoting NCO recombination pathways in mitotic cells would also have a role during meiosis. From our candidate list—*fwh1*, *srs2*, *rgh1*, *fml1*, and *fml2*—only the deletion of *fml1* gave the expected increase in CO formation associated with a meiotic gene conversion (GC) event at two different hotspot alleles, *ade6-M26* and *ade6-3083*, and at a non-hotspot allele *ade6-M375* (Fig. 1, B and C, and tables S1 to S3) (2–5). Increases in COs were also observed on a different chromosome (Fig. 1D and table S4) and by a physical assay at the *mbs1* locus (fig. S1), indicating that Fml1's role in suppressing CO formation is not restricted to a single locus.

In vitro purified Fml1, like its budding yeast ortholog Mph1, unwinds D loops and is therefore suited to promoting SDSA (Fig. 1E) (6, 7). The *fml1-K99R* mutant, which encodes protein that retains full DNA binding activity but is unable to unwind D loops (Fig. 1E and fig. S2), exhibits the same hyper-CO phenotype as the null mutant, indicating that Fml1's helicase function is required for NCO formation (Fig. 1C). A significant increase in CO is also observed by deleting Fml1's cofactors Mhf1 and Mhf2, whose orthologs in humans promote the DNA binding and catalytic activities of Fanconi anemia complementation group M (FANCM) (Fig. 1C and table S2) (8, 9).

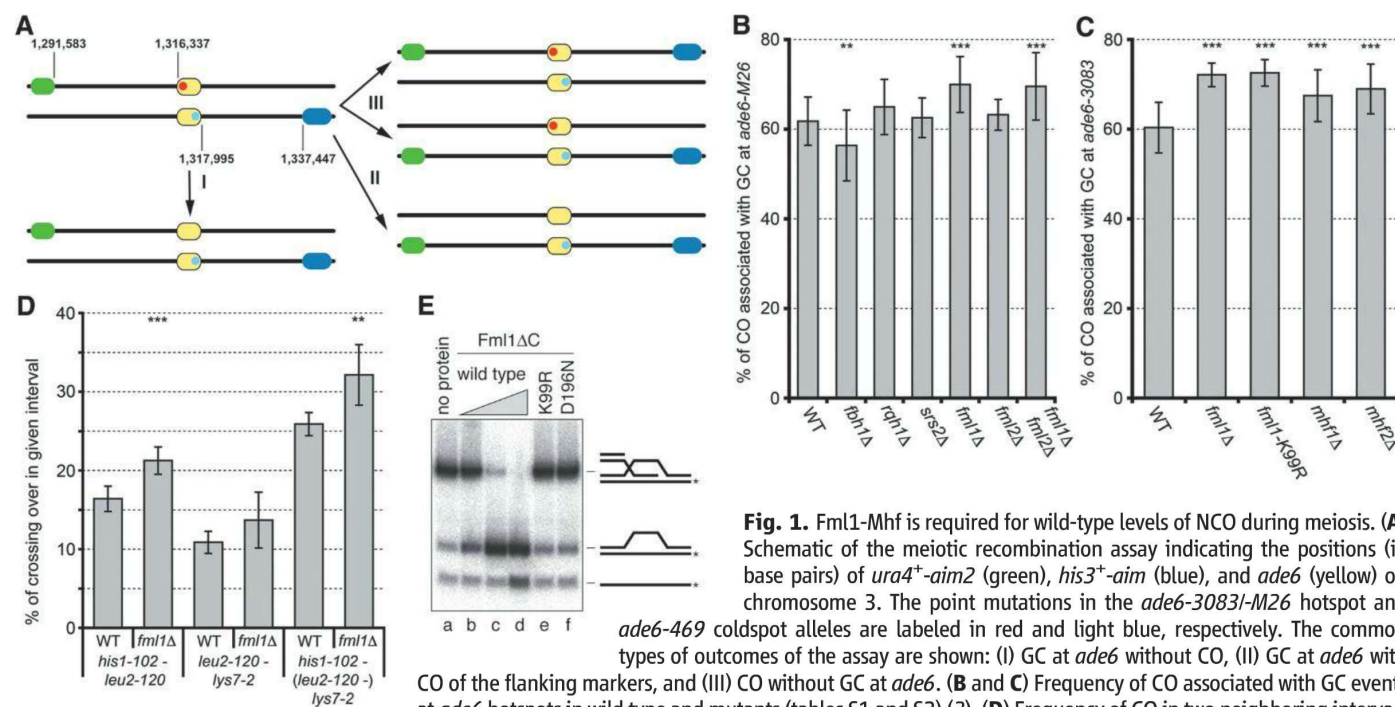


Fig. 1. Fml1-Mhf is required for wild-type levels of NCO during meiosis. (A) Schematic of the meiotic recombination assay indicating the positions (in base pairs) of *ura4⁺-aim2* (green), *his3⁺-aim* (blue), and *ade6* (yellow) on chromosome 3. The point mutations in the *ade6-3083-M26* hotspot and *ade6-469* coldspot alleles are labeled in red and light blue, respectively. The common types of outcomes of the assay are shown: (I) GC at *ade6* without CO, (II) GC at *ade6* with CO of the flanking markers, and (III) CO without GC at *ade6*. (B and C) Frequency of CO associated with GC events at *ade6* hotspots in wild type and mutants (tables S1 and S2) (2). (D) Frequency of CO in two neighboring intervals in wild type and the *fml1Δ* mutant (table S4). In (B) to (D), statistical significance in comparison with wild type is indicated as * $P < 0.1$, ** $P < 0.05$, and *** $P < 0.01$ (for P values, see corresponding tables in the supplementary materials). (E) D loop unwinding by Fml1ΔC (lanes b to d: 0.05, 0.5, and 5 nM), Fml1ΔC-K99R (lane e: 5 nM), and Fml1ΔC-D196N (lane f: 5 nM). The schematics represent the D loop and its dissociation products, with the asterisk indicating the position of the 5' end 32 P label.

In fission yeast, the formation of CO products from joint DNA molecules depends on the endonuclease Mus81-Eme1 (10). The deletion of *mus81* causes joint DNA molecules to remain unresolved, which prevents chromosome segregation and re-

sults in a reduction in the viability of progeny (Fig. 2, A and B, fig. S3, and table S5). The mating efficiency of *mus81Δ fml1Δ* double mutants is very low (table S6), preventing comprehensive genetic analysis; however, visual inspection

of *mus81Δ fml1Δ* asci showed a higher incidence of clumped DNA masses than in *mus81Δ* single mutants, indicating an aggravation of the chromosome segregation problem (Fig. 2B and table S7). These data indicate that at best, Fml1 only poorly substitutes for the loss of the CO recombination pathway by feeding joint molecules into a NCO pathway. The meiosis-specific Rad51-paralogue Dmc1 has been shown to form D loops, which are more resistant to dismantling by DNA translocases than those formed by Rad51 (13); however, in fission yeast deletion of *dmc1* does not change the level of COs associated with GCs (table S2). The Rad51/Dmc1-mediator complex Swi5-Sfr1 (14) is required for wild-type levels of CO, and its deletion ameliorates the defects seen in a *mus81Δ* mutant (Fig. 2, A, B, and C) (15). This rescue of *mus81Δ* by *sfr1Δ* and the reduction of CO formation associated with GC in a *sfr1Δ* single mutant depend on the presence of *fml1* (Fig. 2, A, B, and C). This suggests that Swi5-Sfr1 protects D loops from being unwound by Fml1 and in doing so promotes Mus81-mediated CO formation. In accordance with this, we see a reduction in Mus81 foci in *sfr1Δ* meiotic nuclei compared with wild type (fig. S4 and table S8).

Under vegetative growth conditions, *mus81Δ fml1Δ* strains display synthetic sickness (6), and therefore to confirm that the phenotypes we observe during meiosis are caused by the failure to process meiotic recombination intermediates, we abrogated meiotic DSB formation by deleting *rec12* (also termed *spo11*) in *mus81Δ sfr1Δ*, *mus81Δ fml1Δ*, and *mus81Δ fml1Δ sfr1Δ* strains. The spore viabilities of the mutant combinations were higher than or similar to the 12.5% expected from random segregation of three chromosome pairs (Fig. 2D). Although the spore viability in the *mus81Δ fml1Δ rec12Δ* and *mus81Δ fml1Δ sfr1Δ rec12Δ* crosses is not completely restored to *rec12Δ* levels, the rescue is robust enough to attribute much of the meiotic failure of these mutant combinations to a breakdown in processing meiotic recombination intermediates.

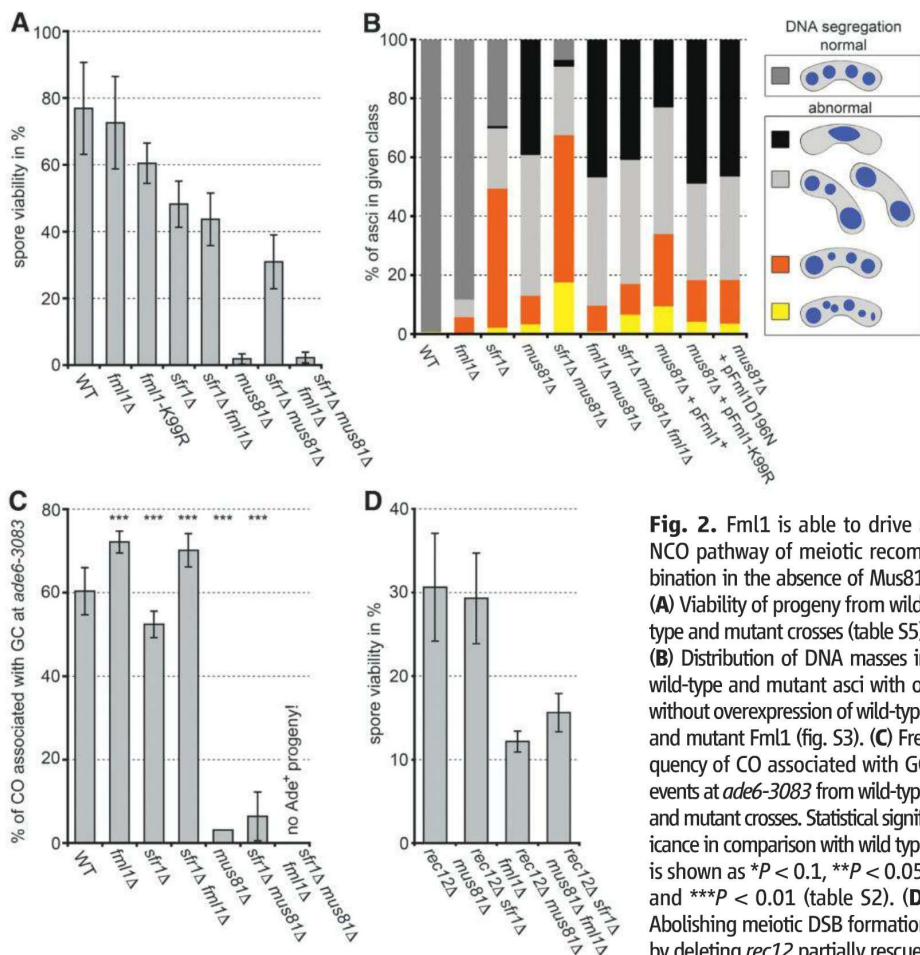
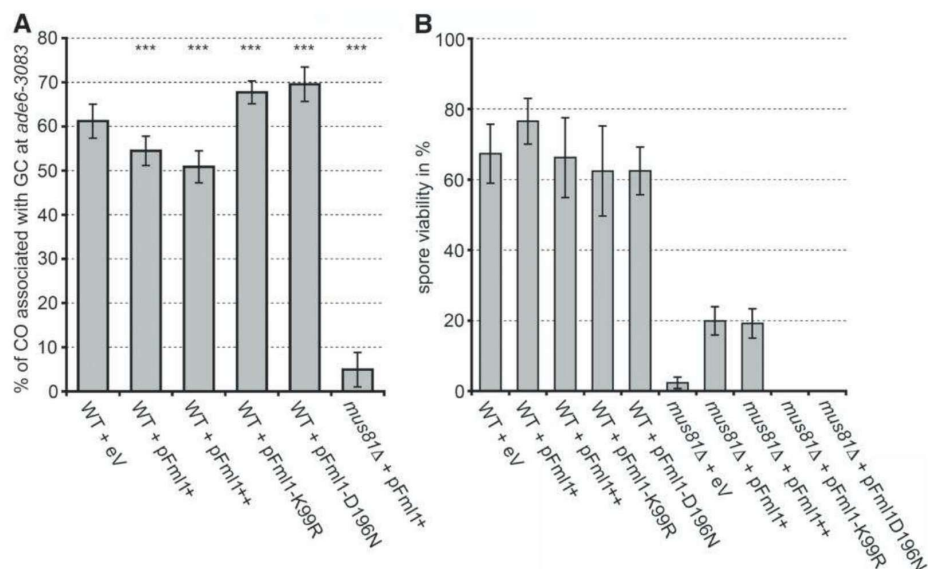


Fig. 2. Fml1 is able to drive a NCO pathway of meiotic recombination in the absence of Mus81. **(A)** Viability of progeny from wild-type and mutant crosses (table S5). **(B)** Distribution of DNA masses in wild-type and mutant asci with or without overexpression of wild-type and mutant Fml1 (fig. S3). **(C)** Frequency of CO associated with GC events at *ade6-3083* from wild-type and mutant crosses. Statistical significance in comparison with wild type is shown as * $P < 0.1$, ** $P < 0.05$, and *** $P < 0.01$ (table S2). **(D)** Abolishing meiotic DSB formation by deleting *rec12* partially rescues the spore viability defect of *mus81Δ fml1Δ* mutants (table S5).

Fig. 3. Overexpression of Fml1 suppresses COs and partially rescues the poor spore viability of a *mus81Δ* mutant. **(A and B)** Frequency of CO associated with GC events at *ade6-3083* (A) and viability of progeny (B) in wild-type and *mus81Δ* crosses overexpressing wild-type and mutant Fml1 (tables S5 and S9). Statistical significance in comparison with wild type in (A) is shown as * $P < 0.1$, ** $P < 0.05$, and *** $P < 0.01$ (for exact P values, see table S9).



The transcription of *mus81*, *eme1*, *swi5*, and *sfr1* is up-regulated (by two- to sixfold) at the start of meiosis, whereas that of *fml1* is not (16). Therefore, we wondered whether relative changes in the amounts of these proteins could influence whether DSBs are repaired as COs or NCOs. Indeed, Fml1 overexpression in wild type reduces COs at *ade6-3083* in a dosage-dependent manner (Fig. 3A and table S9). This effect depends on Fml1's helicase activity because overexpression of Fml1-K99R or Fml1-D196N, which can bind but not unwind D loops (Fig. 1E and fig. S2), causes a significant increase in COs akin to *fml1Δ* (Fig. 3A and table S9). Overexpression of these mutants also confers *fml1Δ*-like sensitivity to genotoxins (fig. S5). Most likely, these mutant proteins impede en-

dogenous wild-type Fml1 and in doing so generate a *fml1Δ*-like phenotype.

Further evidence that the relative amount of Fml1 and Swi5-Sfr1 is a determinant in Fml1's ability to unwind D loops in vivo comes from analyzing the effect of Fml1 overexpression in *mus81Δ* crosses. Here, both the spore viability and chromosome segregation defects of *mus81Δ* crosses are ameliorated in a helicase-dependent manner and in a similar way as deleting *sfr1*: without producing COs (Figs. 2B and 3, A and B). As in wild-type crosses, overexpression of mutant Fml1 probably impedes endogenous wild-type Fml1, worsening the already poor spore viability and chromosome segregation of a *mus81Δ* cross (Figs. 2B and 3B and table S7). The partial rescue of spore viability and chromosome segregation

in *mus81Δ* crosses is specific to Fml1 because none of the other candidate DNA helicases (Rqh1, Srs2, Fbh1, and Fml2) when overexpressed could do this (table S5).

Swapping exogenous Holliday junction (HJ) resolvases, namely bacterial RusA and human GEN1, for Mus81 results in a reduction of CO associated with GC at an *ade6* hot spot from ~60% down to ~40% (17, 18). Our explanation was that these HJ resolvases (in contrast to Mus81-Eme1) cleave recombination intermediates in an unbiased manner, producing COs and NCOs in a 1:1 ratio. We hypothesized that the remaining 20% NCO recombination events stem from SDSA (Fig. 4A). If this is true, then exchanging Mus81 for RusA or GEN1 in a *fml1Δ* background, in which SDSA is abolished, would result in 50% COs and NCOs via unbiased HJ resolution (Fig. 4A). Indeed, 50% COs is what we find when RusA or GEN1 are expressed in *mus81Δ fml1Δ* strains (Fig. 4B).

It is conceivable that the Fml1-dependent NCO pathway proceeds via biased HJ cleavage rather than SDSA. However, deletion of the two known junction-specific nucleases (Slx1 and the XPF ortholog Rad16), which could potentially fulfill this function, has no effect on CO formation or spore viability in a *mus81Δ sfr1Δ* mutant (tables S2 and S5).

Our data show that Fml1-Mhf works in parallel with Mus81-Eme1 to process meiotic joint DNA molecules and that Fml1's ability to produce NCOs is mitigated by a relative up-regulation of a Swi5-Sfr1 and Mus81-Eme1-dependent pathway, in which Swi5-Sfr1 may stabilize Rad51/Dmc1-mediated single-end invasions so that they can be preferentially cleaved by Mus81-Eme1. Fml1 represents the only factor directly driving a meiotic NCO-specific pathway; however, other DNA helicases, such as RTEL-1 in *C. elegans*, apparently can direct the recombination outcome via template choice, creating an additional level of regulation (19, 20).

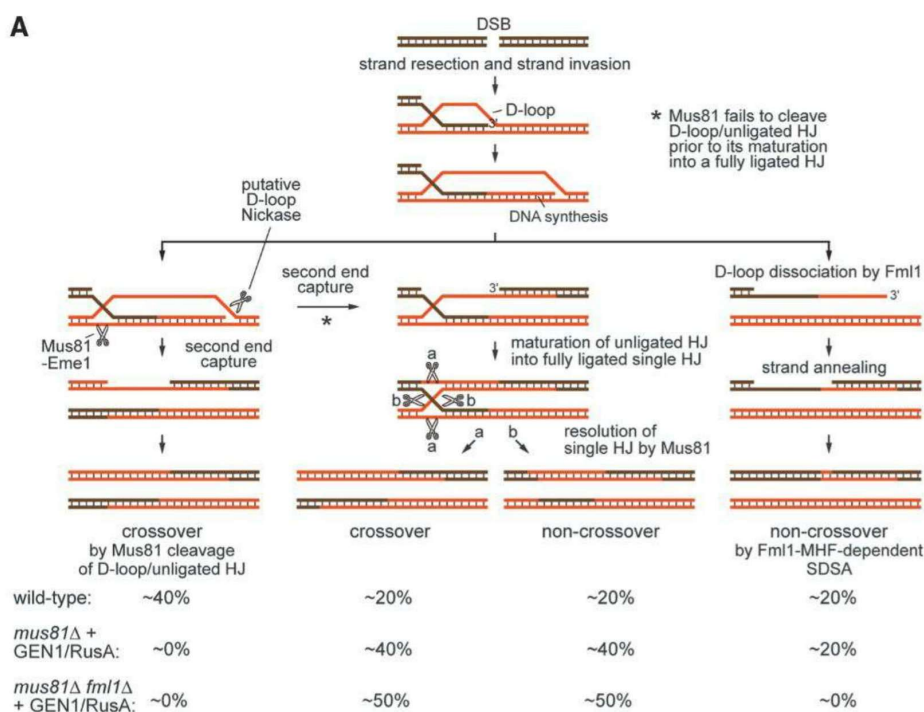


Fig. 4. Meiotic interhomolog recombination pathways in *S. pombe*. **(A)** The respective contribution of recombination pathways to the CO/NCO outcome and the changes observed when a pathway is deactivated. This model accounts for the fact that in a *mus81Δ* strain, only single HJs are observed to accumulate (10), but therefore it needs to invoke a D loop nickase activity (18). **(B)** Frequency of CO associated with GC events at *ade6-3083* from wild-type, *mus81Δ*, and *mus81Δ fml1Δ* crosses expressing Mus81-Eme1, RusA, or GEN1⁽¹⁻⁵²⁷⁾. Statistical significance in comparison with wild type is shown as **P* < 0.1, ***P* < 0.05, and ****P* < 0.01 (table S9) (18).

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Acknowledgments: We thank R. Mercier for discussions and sharing data before publication. We are grateful to A. Decottignies, J. Kohli, R. J. McFarlane, P. Russell, G. R. Smith, and W. W. Steiner for providing strains/reagents and to C. Bryer and J. Witzki for technical assistance. This work was supported by Wellcome Trust Programme grant 090767/Z/09/Z. A.L. was funded in part by an Erwin Schrödinger Fellowship (J 2489-B03) from the Austrian Science Fund (FWF).

Supplementary Materials

www.sciencemag.org/cgi/content/full/336/6088/1585/DC1
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6 February 2012; accepted 9 May 2012
10.1126/science.1220111

FANCM Limits Meiotic Crossovers

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The number of meiotic crossovers (COs) is tightly regulated within a narrow range, despite a large excess of molecular precursors. The factors that limit COs remain largely unknown. Here, using a genetic screen in *Arabidopsis thaliana*, we identified the highly conserved FANCM helicase, which is required for genome stability in humans and yeasts, as a major factor limiting meiotic CO formation. The *fancm* mutant has a threefold-increased CO frequency as compared to the wild type. These extra COs arise not from the pathway that accounts for most of the COs in wild type, but from an alternate, normally minor pathway. Thus, FANCM is a key factor imposing an upper limit on the number of meiotic COs, and its manipulation holds much promise for plant breeding.

Crossovers (COs) are necessary for balanced chromosome segregation during meiosis. Meiotic recombination is initiated by the formation of double-strand breaks (DSBs), which are repaired to yield COs and noncrossovers (NCOs) (1). Two pathways to CO formation are known: Class I CO formation is subject to positive CO interference, whereby one CO reduces the probability of another nearby CO (2), and depends on a group of proteins called ZMMs in addition to MLH1 and MLH3; and class II COs, which are not subject to interference and depend on MUS81 and EME1/MMS4 (1). Mammals, budding yeast, and *Arabidopsis thaliana* have both classes of COs, but some organisms have only one or the other (2). Factors that limit meiotic CO number must exist because DSBs vastly outnumber COs [e.g., 230 versus ~10 in *Arabidopsis* Col-0 (3)].

To identify meiotic anti-CO factors, we designed a screen based on the idea that mutations that increase CO frequency will restore the fertility of CO-defective mutants. *Arabidopsis zmm* mutants, including *zip4*, lack meiotic COs, causing missegregation of homologs and thus reduced fertility leading to shorter fruit that can be visually discriminated from that of the wild type (3) (Fig. 1). *Arabidopsis zip4* mutants still produce enough seeds, through random chromosome segregation, to perform a screen. *zip4-2* seeds were mutated with ethylmethane sulfonate, and these developed into plants indistinguishable

from *zip4*. However, among ~2000 families obtained by self-fertilization, eight lines segregated plants with increased fertility. The causal mutations were single locus, recessive, and fell into three complementation groups. The first complementation group contained five lines and was further studied here (table S1). Mapping and whole-genome sequencing of one suppressor [*zip4(s)1*] identified a missense mutation in the gene *Atlg35530* as the potential causal mutation (Fig. 2, fig. S2, and table S1). *Atlg35530* is the single *Arabidopsis* homolog of the human *Fanconi*

anemia complementation group M (FANCM) (fig. S2). FANCM is involved in genome stability in various eukaryotes but has no known meiotic function (4, 5). Mutations in *FANCM* were found in the four other allelic suppressors, and the suppressor phenotype was recapitulated with a transferred DNA (T-DNA) insertion in *FANCM* (N620621). Three more allelic mutations in *FANCM* were identified in similar ongoing *zmm* screens (*shoc1* and *msh5*) (Fig. 2 and fig. S2). The identified mutations resulted in amino acid changes, all in well-conserved residues of the helicase domain or in splicing sites (Fig. 2A and fig. S2). The single *fancm* mutants were indistinguishable from wild type in terms of growth and fertility [seeds per fruit: wild type = 66 ± 8 ($n = 52$), *zip4* = 3 ± 2 ($n = 65$), *fancm-1 zip4* = 44 ± 8 ($n = 48$), *fancm-1* = 66 ± 7 ($n = 47$)].

In wild type, each pair of homologous chromosomes is invariably linked by a minimum of one chiasma, the cytological manifestation of meiotic COs, resulting in five bivalents at metaphase I. In *zip4*, *msh5*, or *shoc1*, only ~1.5 bivalents form, leaving the ~3.5 remaining pairs of homologs as univalents (Figs. 1 and 2B). In contrast, bivalent formation in all the suppressors

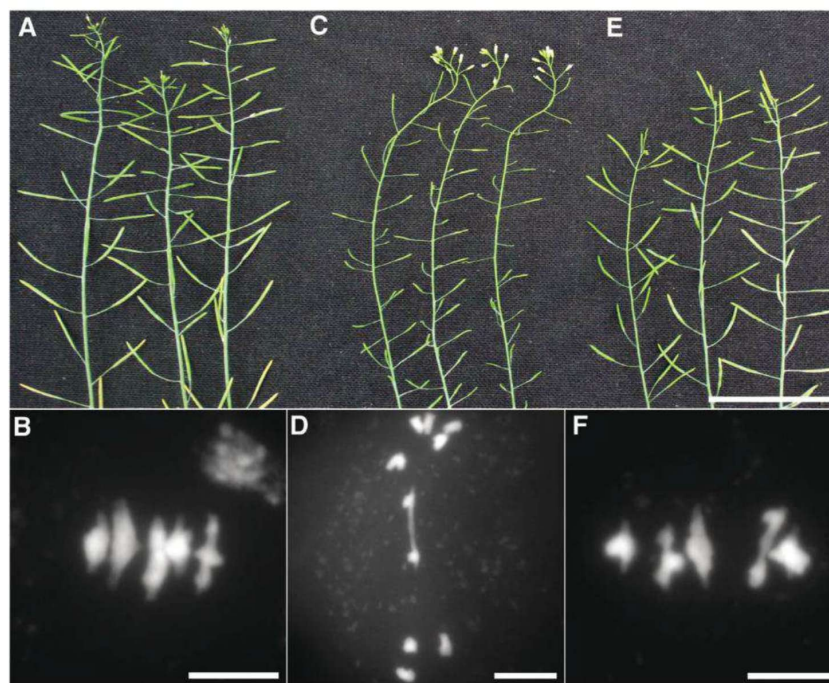


Fig. 1. A screen for genes that limit COs. Wild-type plants have long fruit (A) and five bivalents at metaphase I (B), whereas *zip4* mutants have short fruit (C) and predominantly univalents at metaphase I (D). Suppressors of *zip4* were identified on the basis of increased fruit length (E) and confirmed to have increased bivalent formation (F). Scale bars: (A, C, E) 5 cm; (B, D, F) 5 μ m.

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increases substantially (Fig. 2B), ranging from an average of 4.5 bivalents per cell, to essentially indistinguishable from wild type. A large increase in bivalent formation was also observed in female meiosis in *fancm-1* (fig. S3) and *fancm-5* (not shown). In contrast, *fancm-1* did not restore chiasma formation of *spo11-1* or *dmc1* (Fig. 2), showing that chiasmata arising in *fancm* do not depend on ZMMs but require DSBs and strand invasion catalyzed by SPO11-1 and DMC1, respectively.

We then labeled chromosomes using fluorescence in situ hybridization and examined the shape of metaphase I bivalents (fig. S4). This technique determines if there is zero or at least one chiasma on each chromosome arm. In *zip4*, a majority of chromosomes received no chiasma, whereas in wild type, *fancm-1 zip4*, and *fancm-1*, a majority of chromosomes received at least one chiasma on each arm (table S2). This confirms that chiasma frequency largely increased genome wide when *fancm* was mutated in *zip4*, to a level approaching the upper limit of chiasma number that can intrinsically be detected with this technique. Recombination was therefore measured in a series of genetic intervals by tetrad-based analysis of fluorescent-tagged lines (6, 7) (Fig. 3 and table S3). Recombination was decreased in all tested intervals by a factor of 2 to 3 in *zip4* compared to wild type, consistent with previous findings (3). In *fancm-1 zip4* the genetic distances were increased, by a factor of 1.9 to 3.1 compared to wild type ($P < 10^{-5}$). This confirms that the *FANCM* mutation not only restores CO formation in the absence of ZIP4, but also boosts CO frequency far above that of wild type. In *fancm-1* (with a functional ZIP4), recombination was increased even more than in *fancm-1 zip4*, by an average of 12% ($P < 0.05$ in three individual intervals among six). Thus, when comparing *fancm-1* to wild type, the genetic distance was increased in all eight intervals tested, by a

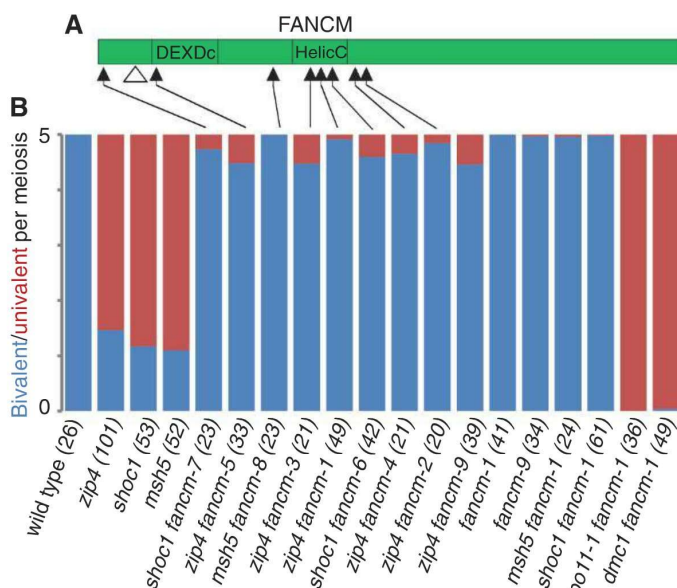
factor ranging from 2 to 3.6 ($P < 10^{-8}$), which highlights the importance of *FANCM* in placing an upper limit on CO frequency.

The difference in CO frequency between *fancm-1* and *fancm-1 zip4* is rather limited compared to the difference between *fancm-1 zip4* and wild type, suggesting that the frequency of ZMM COs is not increased by the absence of *FANCM*. This was further supported by the result that the number of MLH1 foci, a marker of ZMM-dependent chiasmata (8), was identical in wild type and *fancm-1* [11.8 ± 1.6 ($n = 38$) and 11.5 ± 2.1 ($n = 49$), $P = 0.5$] (Fig. 4, A and B). Furthermore, the chiasmata observed in *fancm-1 zip4* are not marked by MLH1 (Fig. 4C). Also, DMC1 foci number is unchanged in *fancm-1* (222 ± 52 , $n = 16$) compared to wild type (215 ± 37 , $n = 14$, fig. S6) (3). We cannot exclude the possibility that mutating *FANCM* does not also increase DSBs, although the unchanged number of DMC1 foci does not support this.

If the extra COs produced in the absence of *FANCM* arise through a non-ZMM pathway, as suggested by the above data, they should be insensitive to interference (2, 9). We measured interference between adjacent intervals (tables S4 and S5) using the interference ratio (6, 7) (table S4), which indicated strong interference in four pairs of intervals in wild type ($P < 3 \times 10^{-4}$). In contrast, in *fancm* and *fancm zip4*, these ratios were not different from 1 (no interference), but were different from the wild-type ratios ($P < 9.10^{-3}$), indicating an absence of interference (table S4). Interference was also measured within each interval by calculating the nonparental ditype ratio (10), which confirmed the absence of detectable interference in *fancm zip4* (table S5).

The extra COs arising in *fancm* are independent of ZIP4, not marked by MLH1, and not sensitive to interference. These characteristics are reminiscent of MUS81-dependent COs, which are a minor fraction in wild type (11, 12).

Fig. 2. *FANCM* mutations restore bivalent formation in *zmm* mutants. (A) The *FANCM* protein with identified mutations indicated as arrows (table S1). The open triangle indicates the position of the *fancm-9* T-DNA insertion. (B) Average number of bivalents (blue) and pairs of univalents (red) per male meiocyte. Number of cells analyzed is indicated in parentheses.



Therefore, we tested whether the extra COs were MUS81-dependent, by introducing the *mus81* mutation into *fancm zip4*. Notably, *fancm mus81* or *fancm mus81 zip4* mutants have a strong growth defect. The *fancm mus81* growth defect was suppressed by mutating *RAD51* (Fig. 4, D to I). This suggests that MUS81 and *FANCM* have redundant roles in homologous recombination during somatic DNA repair.

Despite the growth defect, meiocytes could be obtained from *fancm zip4 mus81* and *fancm mus81* plants. Although meiosis appears to be normal in the double *fancm zip4* or single *mus81* (Fig. 1F and fig. S7), the triple mutation leads to a meiotic catastrophe, showing an absence of bivalents at metaphase I and chromosome fragmentation at anaphase I (Fig. 4J), further arguing that the extra COs appearing in the *fancm* mutant are MUS81 dependent.

In the *fancm mus81* double mutant, with a functional ZIP4, the chromosome fragmentation defect was still present at anaphase I (Fig. 4K), suggesting that the ZMM pathway cannot repair recombination intermediates that accumulate in the absence of both MUS81 and *FANCM*. However, in *fancm mus81*, unlike in *fancm zip4 mus81*, structures resembling bivalents were seen at metaphase I (Fig. 4L), before fragmentation appeared at anaphase I, suggesting the formation of chiasmata. In addition, a wild-type number of MLH1 foci (8) were observed at diplotene (9.9 ± 2.1 , $n = 33$) and diakinesis (9.6 ± 1.8 , $n = 14$) (fig. S8) in *fancm mus81*, suggesting that the formation

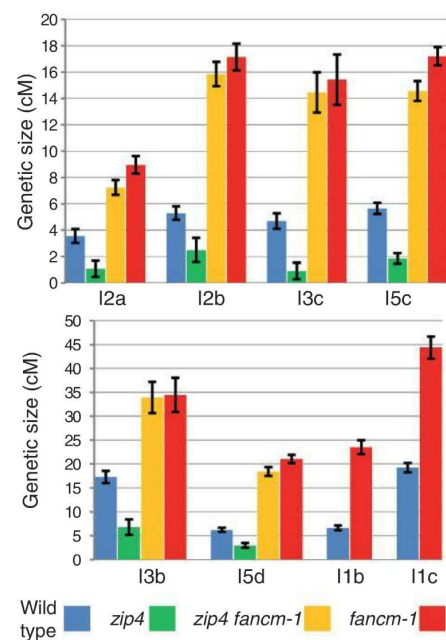


Fig. 3. Meiotic recombination is amplified in the absence of *FANCM*. Genetic distances in eight intervals, measured by using fluorescent-tagged lines, were calculated with the Perkins equation (20) and are given in centiMorgans. I2a and I2b are adjacent intervals on chromosome 2, and so on for the other couples of intervals as described in (6) (fig. S5 and table S3).

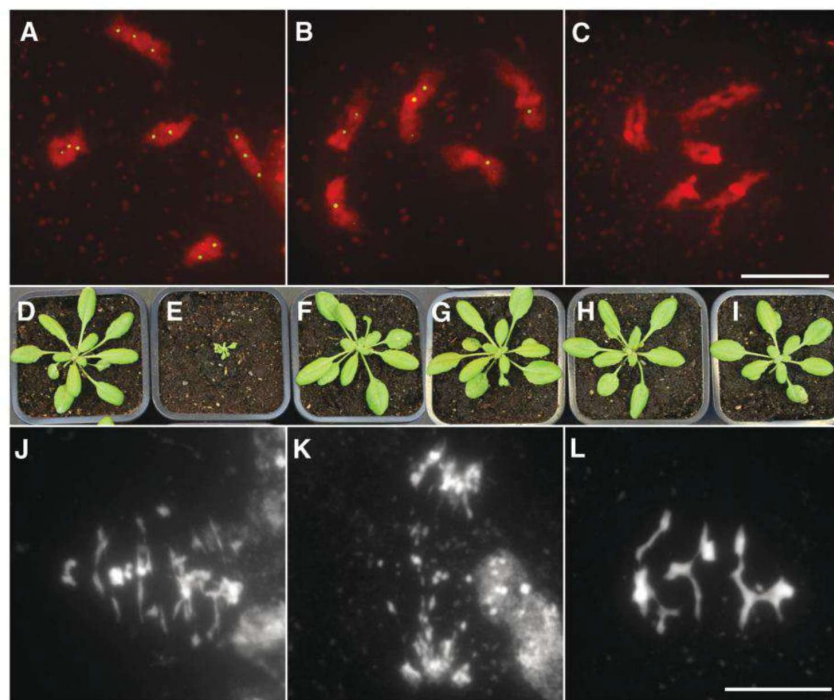


Fig. 4. *fancm* extra COs are MUS81 dependent. (A to C) MLH1 immunolocalization at diakinesis. (A) Wild type. (B) *fancm-1*. (C) *fancm-1 zip4*. (D to I) Analysis of the *fancm mus81* synthetic growth defect. (D) Wild type. (E) *fancm mus81*. (F) *fancm mus81 rad51*. (G) *fancm*. (H) *mus81*. (I) *rad51*. (J to L) Meiotic chromosome spreads. (J) *fancm-1 zip4 mus81* with chromosome fragmentation at the metaphase I to anaphase I transition. (K and L) *fancm-1 mus81* showing chromosome fragmentation at anaphase I and five bivalent-like structures at metaphase I, respectively. Scale bars: 10 μ m.

of ZMM-dependent COs is indifferent to the defective MUS81 FANCM pathways.

In mitotic cells, budding and fission yeast orthologs of the FANCM helicase—Mph1 and Fm11, respectively—have been shown to unwind displacement loops (D loops) in NCO pathways and to be somatic CO suppressors (13, 14). The biochemical properties of FANCM helicases are likely similar at mitosis and meiosis. We suggest that FANCM processes meiotic DSB repair intermediates, possibly D loops, driving them toward NCO resolution (or sister chromatid events) (fig. S1). In the absence of FANCM, MUS81 repairs these intermediates as interference-insensitive COs, whereas ZMMs cannot process these intermediates as COs. This implies that recombination intermediates on which FANCM can act are distinct from the intermediates that ZMMs converts to COs, supporting an early irreversible channeling of intermediates toward the ZMM or MUS81 pathways (9). To date, only two other meiotic anti-CO factors have been described (1): R-TEL in *Caenorhabditis elegans* (15) and Sgs1 in budding yeast (16, 17). Thus, anti-CO factors identified so far are helicases, raising the possibility that different helicases are used in various organisms to prevent excessive COs. Additionally, our findings show that, although most eukaryotes have only one to three COs per chromosome on average, CO number can be largely increased without obvious negative phenotypic effects, suggesting that COs are naturally constrained below their

possible maximum. This finding supports the idea that crossover frequency is maintained by natural selection at a specific equilibrium between the long-term advantages and costs of recombination (18). Finally, the hyperrecombination provoked by *fancm* mutation could be of great interest for crop improvement, which relies on the

production of new allele combinations through meiotic recombination whose frequency is a limiting factor (19).

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Acknowledgments: R.M. and J.L.S. thank the EU-FP7 program (Meiosis-KBBE-2009-222883), and G.P.C. thanks the NSF (MCB-1121563) for financial support. We thank V. Borde, M. Grelon, C. Mézard, F. Nogué, A. Demuyt, and O. Loudet for critical reading of the manuscript and helpful discussions. A provisional patent application based on the work has been filed by INRA.

Supplementary Materials

www.sciencemag.org/cgi/content/full/336/6088/1588/DC1
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References (21–30)

10 February 2012; accepted 17 April 2012
10.1126/science.1220381

Septin-Mediated Plant Cell Invasion by the Rice Blast Fungus, *Magnaporthe oryzae*

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To cause rice blast disease, the fungus *Magnaporthe oryzae* develops a pressurized dome-shaped cell called an appressorium, which physically ruptures the leaf cuticle to gain entry to plant tissue. Here, we report that a toroidal F-actin network assembles in the appressorium by means of four septin guanosine triphosphatases, which polymerize into a dynamic, hetero-oligomeric ring. Septins scaffold F-actin, via the ezrin-radixin-moesin protein Tea1, and phosphatidylinositol interactions at the appressorium plasma membrane. The septin ring assembles in a Cdc42- and Chm1-dependent manner and forms a diffusion barrier to localize the inverse-bin-amphiphysin-RVS-domain protein Rvs167 and the Wiskott-Aldrich syndrome protein Las17 at the point of penetration. Septins thereby provide the cortical rigidity and membrane curvature necessary for protrusion of a rigid penetration peg to breach the leaf surface.

Rice blast is the most devastating disease of cultivated rice and a constant threat to global food security. Each year up to 30%

of the rice harvest is lost to blast disease—enough grain to feed 60 million people—and finding an effective way to control rice blast is therefore a

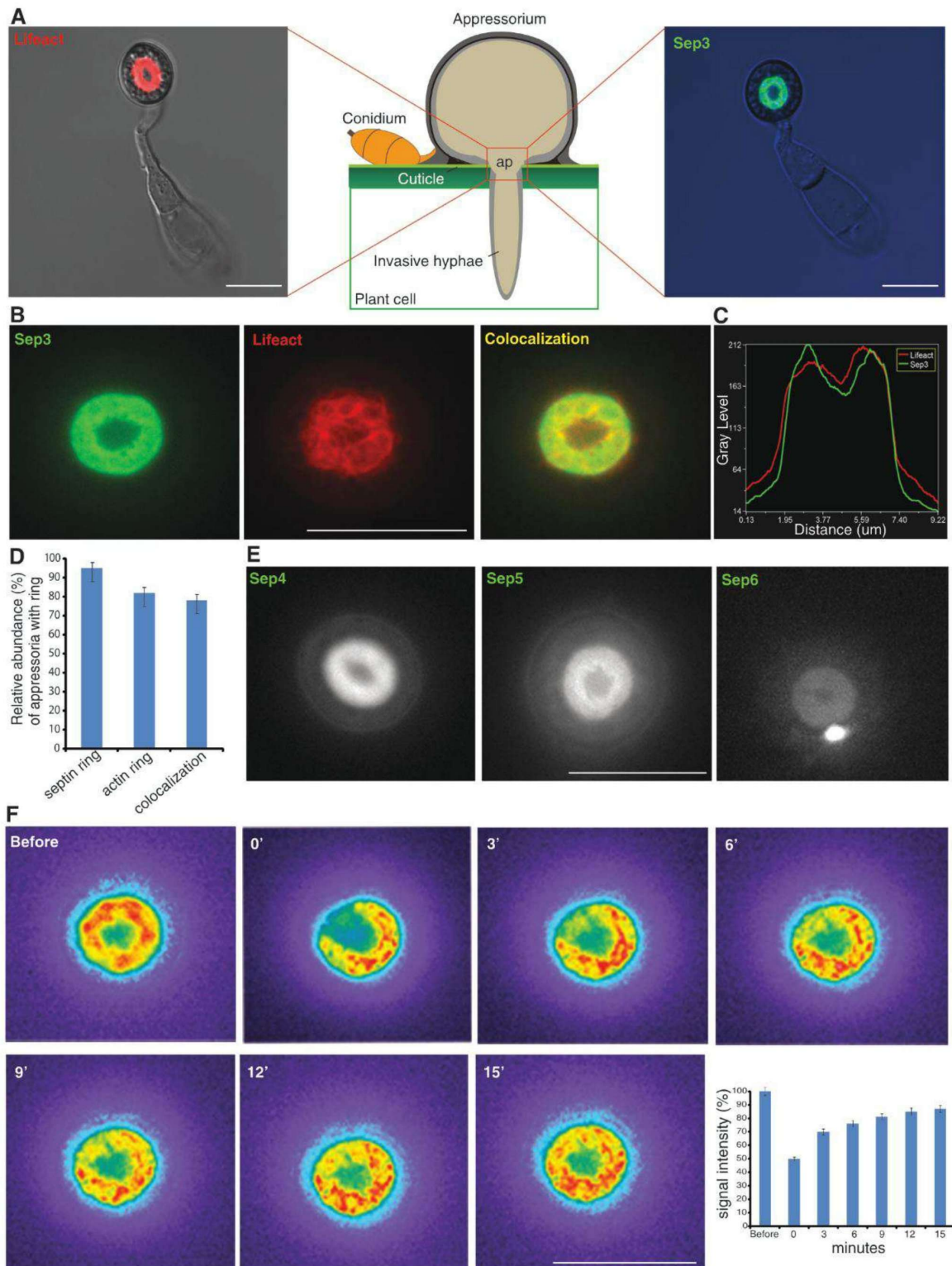
priority. To infect rice leaves, *Magnaporthe oryzae* develops a special infection structure called an appressorium (Fig. 1), which generates turgor of

up to 8.0 MPa (equivalent to 40 times that of a car tire) and translates this extreme pressure into physical force to break the leaf surface. Appressorium turgor is generated by rapid influx of water into the cell against a concentration gradient of glycerol, maintained in the appressorium by a specialized, melanin-rich cell wall (1). Turgor is translated into physical force and applied to the leaf surface by a narrow penetration peg that

mechanically ruptures the tough leaf cuticle (2, 3). The cellular mechanism by which an appressorium breaches the cuticle is not known.

We set out to investigate how the appressorium causes plant infection. We first carried out live-cell imaging of the actin cytoskeleton during appressorium maturation by expressing the actin-binding protein gene fusion, LifeAct-RFP (where RFP is red fluorescent protein) (4), in *M. oryzae*.

Fig. 1. A toroidal-shaped F-actin network and septin ring assemble at the appressorium pore in *M. oryzae*. **(A)** Cellular localization of LifeAct-RFP and Sep3-GFP visualized by laser confocal and laser excitation epifluorescence microscopy, respectively. ap indicates appressorium pore. **(B)** Sep3-GFP and LifeAct-RFP colocalization in the appressorium. **(C)** Linescan graph consistent with colocalization of the septin ring and F-actin network. **(D)** Bar charts showing septin ring and F-actin colocalization (mean $\pm$ SD, three experiments, $n = 300$). **(E)** Sep3-GFP, Sep4-GFP, Sep5-GFP, and Sep6-GFP form a ring at the appressorium pore. Sep6 also forms a distinct punctum. **(F)** Recovery of Sep3-GFP ring after partial photobleaching, with 87% recovery in fluorescence after 15 min (mean $\pm$ SD, three experiments). Scale bars indicate 10 μ m.



This revealed an extensive toroidal F-actin network at the base of the infection cell surrounding the appressorium pore (Fig. 1A), a circular region that marks the point at which the penetration peg emerges to rupture the leaf cuticle (2). The appressorium pore initially lacks a cell wall, and the fungal plasma membrane makes direct contact with the rice leaf surface (2). Then, as the appressorium inflates to full turgor (with a mean diameter of 8.0 μm), a pore wall overlay develops, and a narrow (780-nm mean diameter) penetration peg emerges. Assembly of an F-actin network during appressorium turgor generation, just before plant infection, suggests that specific reorientation of the F-actin cytoskeleton takes place at the base of the appressorium to facilitate plant infection (2). To investigate how actin is organized at this location, we decided to investigate the septin gene family in *M. oryzae*. Septins are small morphogenetic guanosine triphosphatases (GTPases); conserved from yeast to humans (5, 6); and involved in cytokinesis, polarity determination, and secretion (7, 8). Im-

portantly, septins are thought to reorient and reorganize the cytoskeleton to determine cell shape (9–12) and act as partitioning diffusion barriers to recruit and maintain specific proteins at discrete subcellular locations (9, 10). We identified a family of five septin genes in *M. oryzae*, four of which showed similarity to core septins identified in the budding yeast *Saccharomyces cerevisiae* (Cdc3, Cdc10, Cdc11, and Cdc12) (9). Sep3 showed 47% amino acid identity to Cdc3, Sep4 showed 55% identity to Cdc10, Sep5 showed 45% identity to Cdc11, and Sep6 showed 57% identity to Cdc12. We expressed the *M. oryzae* genes in temperature-sensitive *S. cerevisiae* septin mutants that show defects in cell division and in all cases observed complementation of the cell separation defect consistent with the *M. oryzae* proteins acting as functional septins (fig. S1). Next, we expressed Sep3-GFP, Sep4-GFP, Sep5-GFP, and Sep6-GFP (where GFP is green fluorescent protein) gene fusions in *M. oryzae*, and each septin formed a 5.9- μm ring that colocalized with the F-actin network at the appressorium

pore (Fig. 1, A and D, and movie S1). In the case of Sep6-GFP, we also observed an additional bright punctate structure consistently associated with the appressorium septin ring (Fig. 1D). *M. oryzae* septins formed a wider range of structures in hyphae and during invasive growth, including bars, gauzes, collars, and rings (figs. S2 and S3). As expected, they also formed rings at sites of septation (10), such as the neck of nascent appressoria (fig. S2). To understand the nature of the septin ring, we investigated fluorescence recovery after partial photobleaching. We found 87% recovery of fluorescence after 15 min, consistent with the appressorium septin ring being a dynamic structure (Fig. 1F). We then carried out targeted gene deletions to produce isogenic mutants lacking *SEP3*, *SEP4*, *SEP5*, or *SEP6* (fig. S4). Septin null mutants showed mislocalization of the remaining septin-GFPs (fig. S5). Core *M. oryzae* septins therefore act cooperatively to form heterooligomers, which assemble into the large ring surrounding the appressorium pore. Consistent with this idea, coimmunoprecipitation experi-

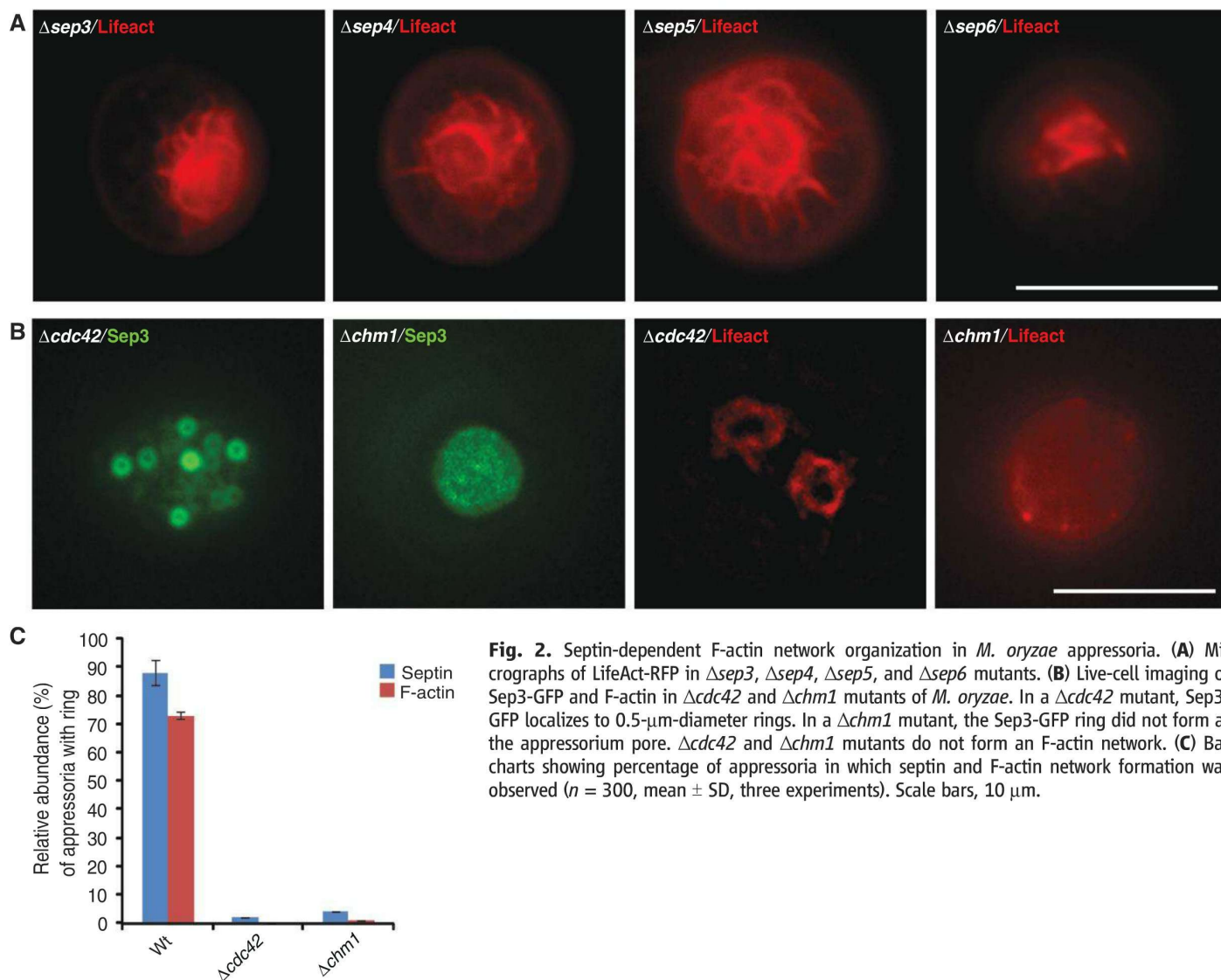


Fig. 2. Septin-dependent F-actin network organization in *M. oryzae* appressoria. (A) Micrographs of LifeAct-RFP in Δsep3 , Δsep4 , Δsep5 , and Δsep6 mutants. (B) Live-cell imaging of Sep3-GFP and F-actin in Δcdc42 and Δchm1 mutants of *M. oryzae*. In a Δcdc42 mutant, Sep3-GFP localizes to 0.5- μm -diameter rings. In a Δchm1 mutant, the Sep3-GFP ring did not form at the appressorium pore. Δcdc42 and Δchm1 mutants do not form an F-actin network. (C) Bar charts showing percentage of appressoria in which septin and F-actin network formation was observed ($n = 300$, mean $\pm$ SD, three experiments). Scale bars, 10 μm .

ments using Sep5-GFP identified Sep3, Sep4, and Sep6 interactions as well as physical interaction with actin, tubulins, and the Lte1 cell cycle control protein (11) (table S1). *M. oryzae* septin mutants showed a number of developmental phenotypes (fig. S6). Multiple rounds of nuclear division, for instance, took place during appressorium development in septin mutants (fig. S7, A and B) compared with a single round of mitosis and autophagy-associated cell death, which normally occurs in *M. oryzae* (12, 13). Septins took part in cytokinesis within hyphae (7–10), and localization of myosin II and myosin light chain to the septum, which separates the germ tube and appressorium (10), required septins (fig. S7, C and D).

We next decided to investigate the effect of deleting *M. oryzae* septin genes on actin organization and found that the appressorium F-actin network was disorganized in $\Delta sep3$, $\Delta sep4$, $\Delta sep5$, and $\Delta sep6$ mutants (Fig. 2A). We reasoned that F-actin, scaffolded by septins, might therefore provide cortical rigidity before peg emergence in a manner analogous to a yeast bud, where assembly of F-actin cables requires Cdc42 and the formins Bni1 and Bnr1 (14–16). To test this idea, we investigated F-actin and septin localization in a *M. oryzae* $\Delta cdc42$ mutant (Fig. 2). We observed many aberrant, ~0.5- μ m-diameter Sep3-GFP rings in a $\Delta cdc42$ mutant but no central septin ring in the appressorium (Fig. 2, B and C).

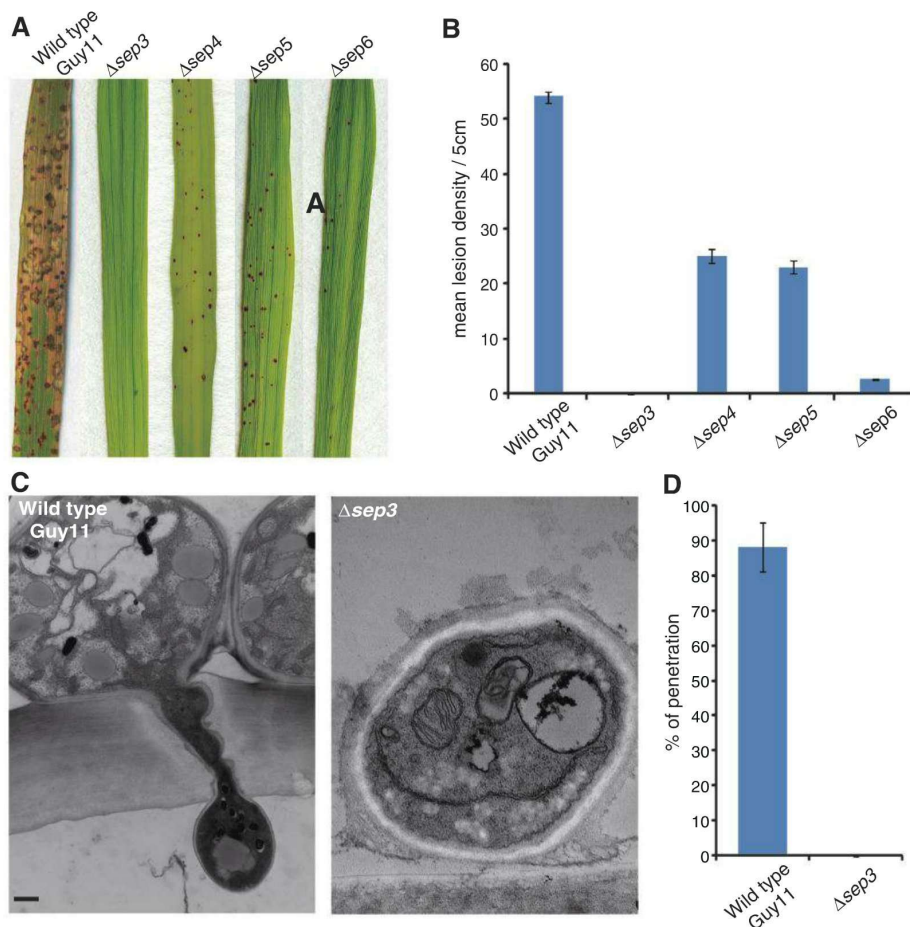
Similarly, targeted deletion of *M. oryzae* *CHM1* (17, 18) prevented formation of either the septin or F-actin networks (Fig. 2, B and C). In yeast, Chm1 encodes a kinase that phosphorylates septins (17). Septin ring formation also required the cell integrity mitogen-activated protein kinase Mps1 (19) and the Mst12 transcription factor (20), which are necessary for appressorium function (fig. S8). Furthermore, we discovered that septin ring formation depends on cell cycle progression, which regulates appressorium development in *M. oryzae* (12) (fig. S9). Taken together, we conclude that septin ring assembly is necessary to scaffold F-actin as a toroidal network at the base of the appressorium before plant infection.

To test whether septin-dependent assembly of the F-actin network is essential for rice blast disease, we inoculated a susceptible rice cultivar with each septin null mutant and scored disease symptoms. Septin mutants were nonpathogenic, causing either no symptoms at all ($\Delta sep3$) or small necrotic flecks associated with abortive infection attempts that stimulate a rice defense response (Fig. 3, A and B). Transmission electron microscopy and live-cell imaging of rice leaf sheath infections by $\Delta sep3$ mutants confirmed the inability of appressoria from septin mutants to rupture plant cuticles efficiently (Fig. 3, C and D). All septin mutant phenotypes were complemented by reintroduction of either a wild-type

allele or the corresponding septin-GFP fusion (fig. S10).

To understand the nature of the cortical F-actin network in appressoria, we decided to investigate plasma membrane linkages at the appressorium base. It has been suggested that some F-actin-plasma membrane linkages may occur via ezrin, radixin, moesin (ERM) proteins, which contain a C-terminal actin-binding domain and an N-terminal ERM domain that binds transmembrane proteins, such as integrins (21). We identified a putative ERM protein-encoding gene in *M. oryzae*, *TEA1* (which showed 61% identity to *S. pombe* Tea1), and found that Tea1-GFP localized as a punctate ring in the appressorium, colocalized with the F-actin and septin networks (Fig. 4A and fig. S11B). By contrast, in a $\Delta sep5$ mutant, Tea1-GFP was mislocalized (Fig. 4A and fig. S11E). Phosphorylation of ERM proteins is potentiated by phosphatidylinositol (PtdIns)-4,5-bisphosphate binding (22). Yeast septins, for example, associate with PtdIns-4-phosphate and PtdIns-4,5-bisphosphate via an N-terminal polybasic domain (23, 24). We identified the *M. oryzae* PtdIns-4-kinase-encoding gene, *STT4* (showing 53% identity to *S. cerevisiae* *STT4*), and the PtdIns-4-phosphate-5-kinase-encoding gene, *MSS4* (64% identity), and found that Stt4-GFP and Mss4-GFP localized to the appressorium pore, bounded by F-actin and the septin ring (Fig. 4B). To test

Fig. 3. *M. oryzae* septin mutants are unable to cause rice blast disease. (A) Targeted deletion of septin genes resulted in loss of pathogenicity on susceptible rice cultivar CO-39. The $\Delta sep3$ mutant caused no disease symptoms; $\Delta sep4$, $\Delta sep5$, and $\Delta sep6$ mutants elicited necrotic flecks because of abortive infection attempts. Septin mutants did not produce spreading disease lesions observed in the isogenic wild-type Guy11. (B) Bar chart shows frequency of disease lesions or necrotic flecks per 5 cm² of leaf surface ($n = 30$ plants) (mean $\pm$ SD, three experiments), reflecting frequency of penetration attempts. (C) Transmission electron micrographs of transverse section of wild-type (24 hours) and $\Delta sep3$ mutant appressoria (36 hours) during leaf infection. No penetration pegs were observed in $\Delta sep3$ mutants. Scale bar, 2 μ m. (D) Bar chart showing frequency of rice epidermal cell rupture at 400 infection sites, 36 hours after conidial germination in wild type (Guy11) and $\Delta sep3$ mutants (mean $\pm$ SD, three experiments).



whether *M. oryzae* septins associated with membrane domains enriched in phosphoinositides, we deleted the N-terminal polybasic domain of *M. oryzae* Sep5 and expressed the resulting Sep5 Δ pb-GFP fusion protein in a Δ sep5 mutant. The Sep5 Δ pb-GFP formed normal septin rings at hyphal septa or the neck of the nascent appressorium, demonstrating that stability of the septin was unaffected by deletion of the polybasic domain (Fig. 4C). However, the septin ring at the base of the appressorium did not form (Fig. 4C). We conclude that septin-PtdIns interactions and ERM protein-actin linkages occur at the appressorium pore. *M. oryzae* Chm1-GFP also localized to the appressorium pore (Fig. 4D and fig. S11, A and F), consistent with a role in septin phosphorylation (25).

We reasoned that, as well as scaffolding F-actin, the septin ring might also act as a diffusion barrier to constrain lateral diffusion of

membrane-associated proteins involved in plant infection. Septin rings are known, for example, to act as diffusion barriers at the mother bud neck in *S. cerevisiae* (26, 27). We decided to test whether *M. oryzae* septins affect distribution of proteins potentially involved in penetration peg development. Bin-amphiphysin-Rvs (BAR) domain proteins have been shown to form oligomeric scaffolds involved in membrane curvature (28). Whereas F-BAR proteins are well known to play roles in membrane invagination during endocytosis, the inverse BAR (I-BAR) proteins are involved in negative membrane curvature leading to cellular protrusions (28). Because emergence of a penetration hypha from the appressorium requires extreme negative membrane curvature (2, 29), we decided to ask whether septins play a role in I-BAR protein localization. *M. oryzae* Rvs167 contains an I-BAR domain, showing 75% identity to *S. cerevisiae* Rvs167p. We found that

Rvs167-GFP localized to the center of the appressorium pore, before penetration peg emergence. However, distribution of Rvs167-GFP was disrupted in a Δ sep5 mutant (Fig. 4E and fig. S11, C and G). I-BAR proteins are proposed to link curved membrane structures to polymerization of cortical F-actin via nonspecific electrostatic interactions (30) mediated by Src homology 3 (SH3) domains of I-BAR proteins and components of the Wiskott-Aldrich syndrome protein (WASP)/WASP-family verprolin-homologous protein (WAVE) complex (31). We isolated a WASP/Arp2/3 complex component, Las17 (32), homologous to Las17p of *S. cerevisiae*. *M. oryzae* Las17-GFP localized to the base of the appressorium, bounded by the septin ring (Fig. 4F), whereas in a Δ sep5 mutant Las17-GFP localization was disrupted, with the protein distributed diffusely in the cytoplasm and plasma membrane (Fig. 4F and fig. S11, D and H).

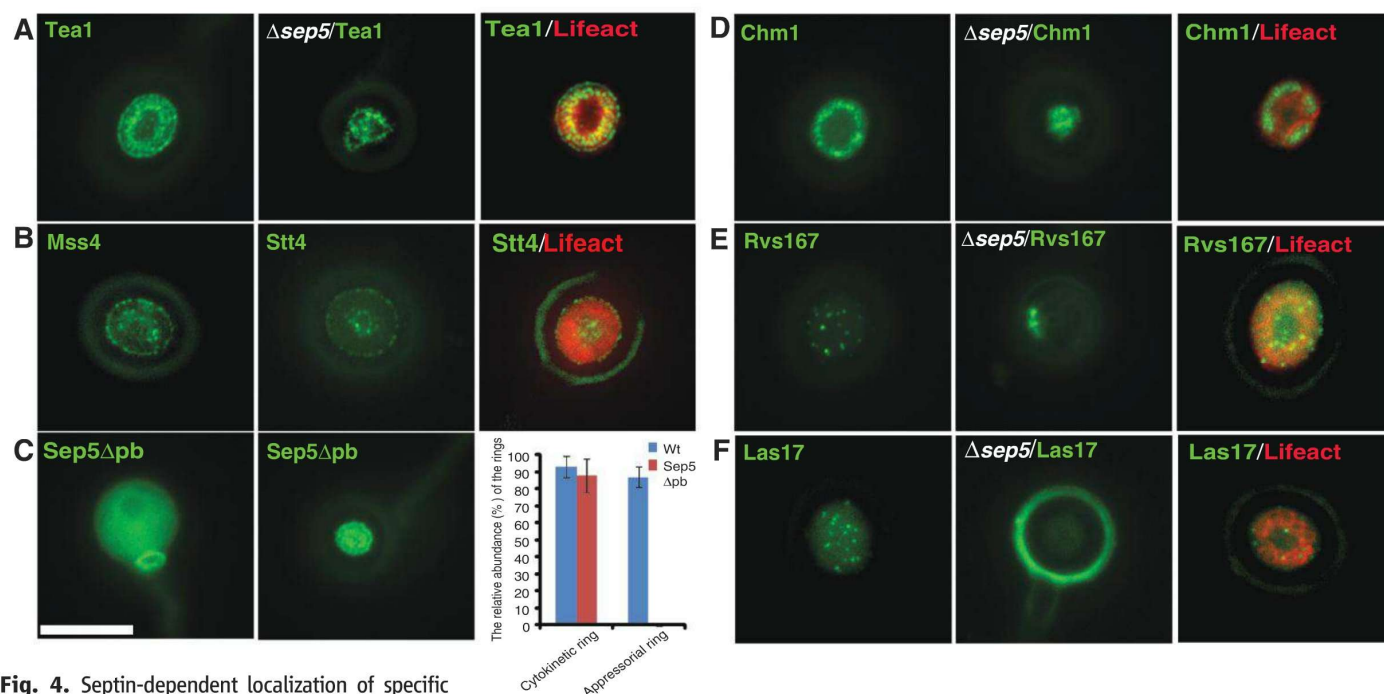


Fig. 4. Septin-dependent localization of specific proteins to the appressorium pore in *M. oryzae* and model of septin-mediated plant infection. (A) Micrographs show localization of Tea1-GFP in Guy11 and in a Δ sep5 mutant and colocalization with LifeAct-RFP (mean $\pm$ SD, three experiments, $n = 300$). (B) Micrographs show PtdIns 4-kinase (Stt4-GFP) and PtdIns-4-phosphate 5-kinase (Mss4-GFP) localization at center and periphery of the appressorium pore and colocalization with LifeAct-RFP. (C) Micrographs and bar chart show that the N-terminal polybasic-rich domain (pb) of Sep5 is dispensable for septin ring formation at site of cytokinesis at appressorium neck (left) but necessary for septin ring formation at the appressorium pore (right). (D) Chm1-GFP colocalizes with LifeAct-RFP, and this is impaired in a Δ sep5 mutant. (E) The I-BAR protein (Rvs167-GFP) localizes to puncta in the appressorium pore, and its organization is affected in a Δ sep5 mutant. (F) Localization of the N-WASP protein (Las17-GFP), a component of the actin-polymerizing Arp2/3 complex, to the center of the appressorium pore septin ring and impairment of its localization in a Δ sep5 mutant. Scale bar, 10 μ m. (G) Model to show septin-mediated rice leaf infection by *M. oryzae*.

On the basis of the evidence presented in this report, we propose that appressoria of the rice blast fungus infect plants by using a septin-dependent mechanism summarized in Fig. 4G. In this model, isotropic expansion of the pressurized appressorium is directed into mechanical force at the base of the infection cell. This is dependent on assembly of an extensive toroidal F-actin network at the appressorium pore to provide cortical rigidity at the initially wall-less region of the appressorium. Septins organize the actin network, making direct phosphoinositide linkages to the plasma membrane and facilitating the action of ERM proteins, such as Teal1, which link cortical F-actin to the membrane. The septin ring also acts as a diffusion barrier to ensure localization of proteins, such as the Rvs167 I-BAR protein, and the WASP/WAVE complex involved in membrane curvature at the tip of the emerging penetration peg and F-actin polymerization (Fig. 4G). In this way, the rice blast fungus extends a rigid penetration peg that ruptures the leaf cuticle and invades the host plant tissue.

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Acknowledgments: We thank J. Thorner (UC Berkeley) for providing yeast septin mutants, M. Momany for critical reading of the manuscript and valuable discussions, H. Florence from Exeter Mass Spectrometry facility, and M. Schuster from the Exeter Bio-imaging Centre. This work was funded by a Halpin Scholarship for rice blast research to Y.F.D. and grants to N.J.T. from the Biotechnology and Biological Sciences Research Council and the European Research Council. K.Y. is funded by the Kyoto University Foundation. Accession numbers are provided in supplementary materials.

Supplementary Materials

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Materials and Methods

Figs. S1 to S12

Tables S1 and S2

References (33–40)

Movie S1

4 April 2012; accepted 15 May 2012

10.1126/science.1222934

The *lac* Repressor Displays Facilitated Diffusion in Living Cells

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Transcription factors (TFs) are proteins that regulate the expression of genes by binding sequence-specific sites on the chromosome. It has been proposed that to find these sites fast and accurately, TFs combine one-dimensional (1D) sliding on DNA with 3D diffusion in the cytoplasm. This facilitated diffusion mechanism has been demonstrated *in vitro*, but it has not been shown experimentally to be exploited in living cells. We have developed a single-molecule assay that allows us to investigate the sliding process in living bacteria. Here we show that the *lac* repressor slides 45 ± 10 base pairs on chromosomal DNA and that sliding can be obstructed by other DNA-bound proteins near the operator. Furthermore, the repressor frequently (>90%) slides over its natural *lacO*₁ operator several times before binding. This suggests a trade-off between rapid search on nonspecific sequences and fast binding at the specific sequence.

Transcription factors (TFs) have evolved to rapidly find their specific binding sites among millions of nonspecific sites on chromosomal DNA (1). In 1970, Riggs *et al.* showed that *in vitro* the *lac* repressor (LacI) finds its operator apparently faster than the rate limit for three-dimensional (3D) diffusion (2). These experiments were explained by the facilitated diffusion theory (3, 4), which posits that TFs search for their binding sites

through a combination of 3D diffusion in the cytoplasm and 1D diffusion (sliding) along the DNA. The sliding effectively extends the target region to the sliding distance, which facilitates the search process. Since then, sliding on DNA has been studied in various *in vitro* assays (5–8), including direct observations in single-molecule experiments (9, 10). However, the physiological relevance of the long sliding distances observed at low salt concentrations *in vitro* has been questioned (11, 12). This is because the high intracellular concentrations of salt and nucleoid proteins are expected to reduce sliding distances (13). Therefore, whether facilitated diffusion is used in living cells, and if so, how

far a TF slides on chromosomal DNA, remain unanswered questions.

Single-molecule imaging provides the time resolution necessary to study TF binding kinetics in living cells. Using a yellow fluorescent protein–labeled LacI in *Escherichia coli* cells, we developed an assay for measuring the search time based on the distinction between localized and diffuse fluorescence signals (14). On a 4-s time scale, individual operator-bound LacI-Venus molecules appear as diffraction-limited spots over the background of freely diffusing molecules (Fig. 1A). By measuring the average number of fluorescent spots per cell as a function of time after removing the inducer isopropyl-β-D-1-thiogalactopyranoside (IPTG) (fig. S7), the kinetics of TF binding to an individual operator site in the *E. coli* chromosome can be monitored at a time resolution of seconds (Fig. 1B) (15). The fusion to Venus prevents LacI from forming tetramers, thus removing the possibility that the protein will loop DNA. The accuracy of the assay depends on limiting the total number of repressors to three to five molecules per cell (14, 16). This can be achieved (fig. S6) by increasing the autorepression through a single artificial *lacO*_{sym} operator site partially overlapping the repressor coding region (Fig. 1B, inset).

When we measured the association rate in the strain with a single *lacO*_{sym} (JE101), we found that it took an average time of 56 ± 2 s (SEM) for any of the three to five fully active (supplementary text) LacI-Venus dimers to bind the operator site (Fig. 1B). This implies that the time required

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for a single repressor molecule to bind *lacO_{sym}* is 3 to 5 min. These observations are in excellent agreement with recent theoretical binding-time predictions (3.5 min) based on facilitated diffusion by sliding (13) (supplementary text). This overall agreement suggests the possibility of a sliding mechanism, without proving any of the specific assumptions made in the theoretical model.

To directly evaluate whether the *lac* repressor slides on nonspecific DNA sequences in vivo—and if so, how far—we made strains (15) with two identical *lac* operator sequences separated by different distances (Fig. 2A, fig. S5, and table S1) and measured how fast the *lac* repressor finds any one of these sites (Fig. 2B). This is an in vivo version of the in vitro assay for TF sliding developed by Ruusala and Crothers (7). The rationale is that if the distance between two operator sites is smaller than the sliding distance, they will appear as one search target, whereas two distant operator sites will appear as two independent targets. Indeed, when the two operator sites were positioned 115 base pairs (bp) or 203 bp apart, the first binding event of the two operators occurred twice as fast as the binding event of a single operator (Fig. 2B and fig. S8). This implies that the two operators are perceived as independent targets in the search process and that the sliding distance is shorter than 115 bp. However, when the distance between the operator sites was shortened to 45 or 25 bp, there was a significant decrease in the rate of binding (Fig. 2B), demonstrating that the target regions of the two operators partly overlap. This suggests that the effective target region of an individual operator site is much larger than the ~1 bp precision needed for specific binding by 3D diffusion only (3).

To calculate the size of the target region, we derived the association-rate dependence on the operator distance based on the facilitated diffusion model (3) (supplementary text and fig. S3). The rate of binding in the two-operator case, r_2 , in relation to the rate of binding in the one-operator case, r_1 , can be expressed as

$$\frac{r_2}{r_1} = 1 + \tanh(L/2s) \quad (1)$$

where L is the center-to-center distance between the operators and $s = \sqrt{D/k_d}$ is the effective capture distance along DNA from each side of one operator. In deriving this relationship, it is assumed that the TF slides along a DNA segment at rate D ($\text{bp}^2 \text{s}^{-1}$), that it dissociates from the DNA at rate k_d (s^{-1}), and that the TF always binds as soon as it reaches the operator. The root mean square sliding distance is $s_L = s\sqrt{2}$. A best fit of the association rates in Fig. 2B to Eq. 1 gives an effective sliding distance $s_L = 36 \pm 6$ bp (SEM).

One consequence of sliding on DNA before binding to a specific site is that another protein bound next to the operator would block sliding from that side and therefore reduce the overall association rate by up to a factor of 2. To test this

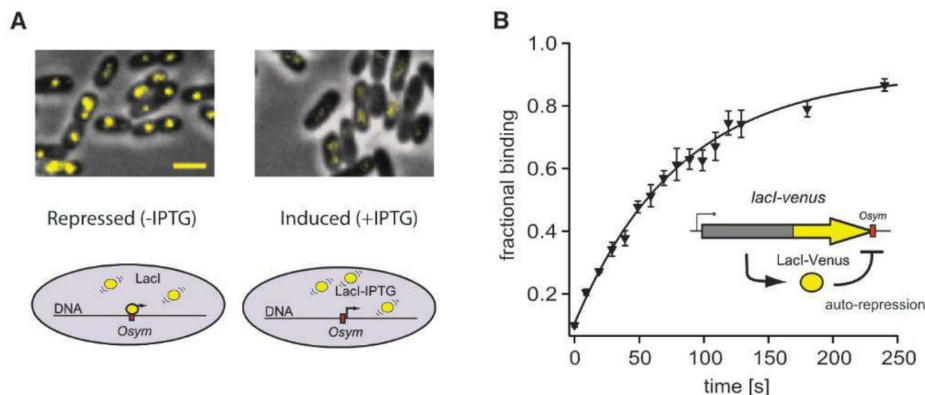


Fig. 1. Single-operator binding assay. **(A)** Overlays of *E. coli* cells imaged in phase contrast and fluorescence microscopy. (Left) Repressing state (–IPTG); LacI-Venus binds the single chromosomal operator (*lacO_{sym}*) and appears as a diffraction-limited spot (yellow). (Right) The cells are induced with IPTG (300 μM), which prevents specific operator binding and results in a diffuse fluorescence signal from the rapidly diffusing LacI-Venus molecules. Scale bar: 2 μm . **(B)** The association rate to a single chromosomal operator is determined by studying the rebinding kinetics of the repressor to the operator after removing the inducer. The fractional binding, i.e., the average number of bright diffraction-limited spots per cell as a function of time, is fitted to the single exponential function $a(1 - be^{-kt})$. (Inset) The accuracy of the assay depends on maintaining a low and even expression of LacI-Venus. This is achieved by using strong autorepression mediated by the ideal *lac* operator *lacO_{sym}*, or *lacO₁* in a position that overlaps the 3' end of the *lacI-venus* coding sequence (sequence level information is in table S1).

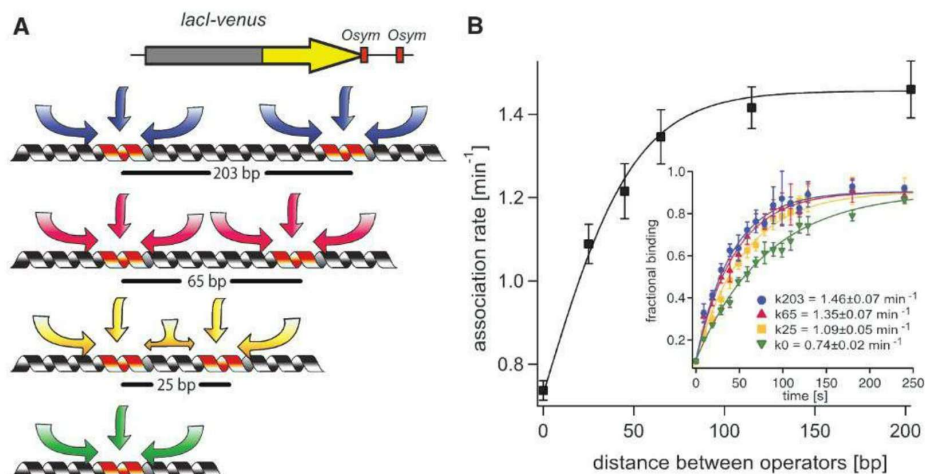


Fig. 2. Sliding-length determination. **(A)** Two identical *lacO_{sym}* operator sites are positioned at center-to-center distances 25, 45, 65, 115, or 203 bp (45 and 115 bp not shown) in different strains. The capture region of each operator is indicated with colored arrows. When the operators are far apart, they are expected to be independent targets in the search process. When the operators are moved closer, such that the capture regions of two operators overlap, they should appear more and more as one operator site (green). The strains express the same amount of LacI-Venus, as shown by Western blots (fig. S6). **(B)** Rate of binding to the first of two identical operator sites as a function of the interoperator distance. The association rates are fitted to the theoretical sliding length dependence curve (black line), $c(1 + \tanh(L/(s_L\sqrt{2})))$, where L is the distance between operators. $c = 0.73 \pm 0.02 \text{ min}^{-1}$ is the association rate to a single operator, and $s_L = 36 \pm 6$ bp is the sliding distance. Error bars indicate $\pm$ SEM; $n \geq 8$. (Inset) Individual association rate measurements (data for 45 and 115 bp are provided in fig. S8).

prediction, we placed the operator *tetO₂* of the transcription factor TetR next to a single *lacO_{sym}* operator (Fig. 3A). We optimized the position of the *tetO₂* site by using molecular-dynamics simulations to place the TetR as close as possible to LacI without allowing contact between the bound proteins (Fig. 3C). When TetR was present, the rate of LacI binding to *lacO_{sym}* was reduced by

a factor of 1.75 ± 0.18 when the *tetO₂* site was next to the *lacO_{sym}* site as compared to when there was no *tetO₂* site (Fig. 3A). However, the equilibrium binding of LacI to *lacO_{sym}* was not changed by TetR binding (Fig. 3, A and B, and fig. S14). Thus, TetR obstructs the entry and exit pathways of the *lac* operator without changing the free energy of the bound state.

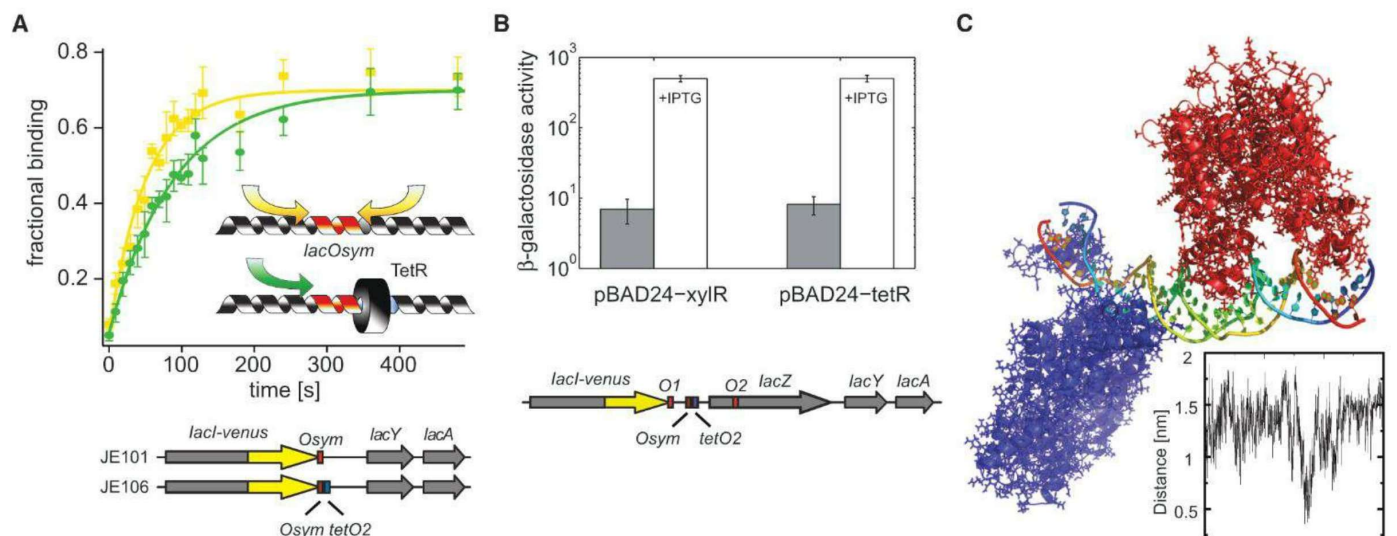


Fig. 3. A TetR roadblock reduces the association rate of LacI without changing its equilibrium binding. **(A)** Single-operator binding kinetics without (yellow squares) and with (green circles) a *tetO₂* operator site juxtaposed to the *lacO_{sym}* operator. TetR is expressed from plasmid pQE30 in both strains. The association rates are $1.18 \pm 0.09 \text{ min}^{-1}$ without *tetO₂* and $0.68 \pm 0.05 \text{ min}^{-1}$ with *tetO₂* (the ratio is 1.75 ± 0.18). Error bars indicate $\pm \text{SEM}$; $n \geq 4$. **(B)** β-Galactosidase activity assay showing how the regulation of *lacZ* is influenced by TetR binding next to the *lacO_{sym}* as in (A). TetR or XylR is expressed from plasmid pBAD24. **(C)** Molecular-dynamics simulation of LacI (blue) and TetR (red) binding on DNA in the same configuration as in (A) and (B). The simulations are based on crystallographic data of LacI and TetR binding their respective operator. The snapshot image illustrates how the two transcription factors are separated in space. The inset shows how the shortest distance between the proteins changes over time in the simulation.

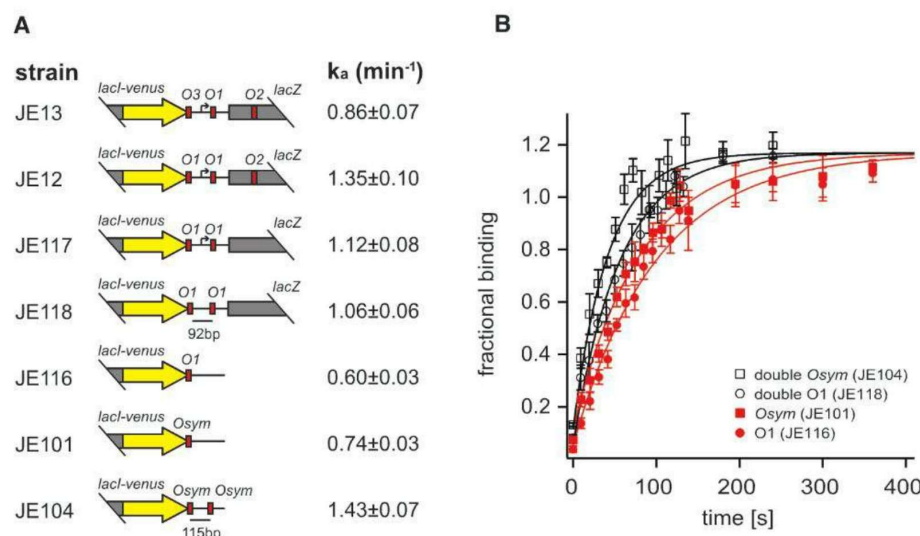


Fig. 4. Contributions from the elements of the wild-type operator region. **(A)** Association rates measured for strains stepwise modified from the wild-type operator configuration in JE13. The arrow indicates the promoter region with binding sites for CRP, H-NS, and RNAP. The contributions of individual operators fit an additive model with *lacO_{sym}* = 0.73 min^{-1} , *lacO₁* = 0.57 min^{-1} , *lacO₂* = 0.25 min^{-1} , and *lacO₃* = 0.06 min^{-1} . See supplementary text for all binding curves and calculations. Error bars indicate $\pm \text{SEM}$; $n \geq 4$. **(B)** Comparison of association rates for *lacO₁* and *lacO_{sym}* operators. Binding curves are shown for a single *lacO₁* (red filled circles), a single *lacO_{sym}* (red filled squares), two *lacO₁* (black open circles), and two *lacO_{sym}* (black open squares). Association rates [in (A)] are given by single exponential fits (supplementary text). Error bars indicate $\pm \text{SEM}$; $n \geq 4$.

The wild-type *lac* operator region is more complicated than in the simplified constructs. It includes, for example, the binding sites for RNA polymerase (RNAP), cAMP receptor protein (CRP), and histone-like nucleoid structuring protein (H-NS) (17, 18). This raises the possibility

that these proteins act like the TetR-roadblock and make sliding less important in the natural context than in our constructs. To identify the contribution of each element in the wild-type *lac* operator region, we modified it stepwise into our artificial construct (Fig. 4A). Starting with the

wild-type operator configuration *lacO₃-O₁-O₂* and changing *lacO₃* to the much stronger *lacO₁* operator increased the association rate for the TF to the operator region by a factor of 1.57 (figs. S10, F and H, and S11). Next, the weak *lacO₂* site was removed from *lacO₁-O₁-O₂*. This decreased the rate of binding only by a factor of 1.20 (fig. S10E), implying that the association rate to *lacO₂* is lower than to *lacO₁*. Finally, we replaced the binding sites for RNAP, CRP, and H-NS between the two remaining *lacO₁* sites. This did not change the rate of LacI binding significantly (fig. S10D), showing that the RNAP, CRP, and H-NS sites are not sufficiently occupied to hinder sliding under our experimental conditions.

The rate of binding to the two independent *lacO₁* operators in the modified *lac* operator region (JE118) is 26% smaller than that to the two independent *lacO_{sym}* operators (JE104) in the sliding distance experiment (Fig. 4B). This observation is inconsistent with previous theoretical models (3, 14, 19), which have assumed that the rate of operator binding is diffusion limited and thus independent of variations in operator sequence. The difference was, however, confirmed by comparing association rates to single *lacO₁* and *lacO_{sym}* operators in otherwise identical strains (rates: $0.74 \pm 0.03 \text{ min}^{-1}$ for *lacO_{sym}* and $0.60 \pm 0.03 \text{ min}^{-1}$ for *lacO₁*) (Fig. 4B). The difference implies that the repressor has different probabilities, p_{bind} , of binding the different operators before sliding to the neighboring base pair. There are, however, neither theoretical predictions nor experimental estimates of this probability for any TF. Therefore, we have here rederived the established search rate equations to include the probability of

binding the operator (supplementary text and fig. S1). The difference in association rate between $lacO_{sym}$ and $lacO_I$ means that the upper bound for $lacO_I$ is $p_{bind} = 0.12$, if we consider that the upper bound for $lacO_{sym}$ is $p_{bind} = 1$.

One method to determine the absolute value of p_{bind} is to measure association rates to a *lac* operator in strains where roadblocks for sliding have been placed on both sides of the operator (supplementary text). For $p_{bind} = 1$, the overall association rate would be strongly dependent on the distance between the roadblocks because they would determine the effective target region. A $p_{bind} \ll 1$ would imply that the operator is bypassed several times before binding. This would reduce the impact of the roadblocks because the restricted target region would be largely compensated by an increased number of return events (fig. S2). On the basis of measurements with TetR roadblocks at 7 or 12 bp from the *lac* operator, we estimate that $p_{bind}^{OI} = 0.044$ and $p_{bind}^{O_{sym}} = 0.094$ (supplementary text and fig. S13).

It may seem that this low p_{bind} would make the TF search hopelessly inefficient. However, in terms of search efficiency, one has to take into account that the TF returns many times ($s_L/\sqrt{2}$) before leaving the capture region of the operator DNA. In the refined search model, including the low p_{bind} , the sliding distance is $s_L = 45 \pm 10$ bp and the overall probability of binding a $lacO_I$ operator, including multiple return events, is $53 \pm 24\%$ (supplementary text and figs. S1 and S2). One important consequence of $p_{bind} < 1$ is that it invalidates the classical prediction (3, 19) that the maximal association rate can be achieved when the repressor spends 50% of the time free in the cytoplasm and 50% nonspecifically bound. When the association rate is maximized for $p_{bind} < 1$, the

optimal fraction of time the TF is nonspecifically bound will be larger than 50% (supplementary text). The experimentally observed 90% nonspecific binding (14, 20) is optimal for the lower range of the estimated binding probabilities (supplementary text and fig. S4).

We have demonstrated here that the *lac* repressor slides into its chromosomal operators in living bacterial cells. The average sliding distance is $s_L = 45 \pm 10$ bp, which is close to what is measured in vitro (5, 7) for ionic strengths corresponding to 0.15 M monovalent cations (21). The sliding makes the search for a $lacO_I$ operator 40 times as fast (Eq. S8) as the situation where the repressor binds DNA nonspecifically but does not slide. In relation to a hypothetical situation where the repressor could bind directly to an operator without nonspecific interactions, the speed-up is a modest factor of 4 (Eq. S14), i.e., the in vivo binding is not much faster than the theoretical diffusion limit for 3D diffusion alone. The fact that the sliding TF does not bind to the operator upon first encounter opens a new level of complexity in the evolution of transcription factors and their binding sites. Indeed, it may be physically impossible to combine a high probability of binding at specific sites with a fast search at nonspecific sites.

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Acknowledgments: We are grateful to G.-W. Li, M. Ehrenberg, X. S. Xie, and N. Grantcharova for helpful suggestions and to members of the Elf lab for comments on the manuscript. This work was supported by the European Research Council, the Swedish Foundation for Strategic Research (SSF), the Swedish Research Council (VR), Göran Gustafssons Stiftelse, EMBO young investigator programme, and the Knut and Alice Wallenberg Foundation. A.M. was funded by the Centre for Interdisciplinary Mathematics, Uppsala University.

Supplementary Materials

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Material and Methods
Supplementary Text
Figs. S1 to S14
Table S1
References (22–55)

8 March 2012; accepted 7 May 2012
10.1126/science.1221648

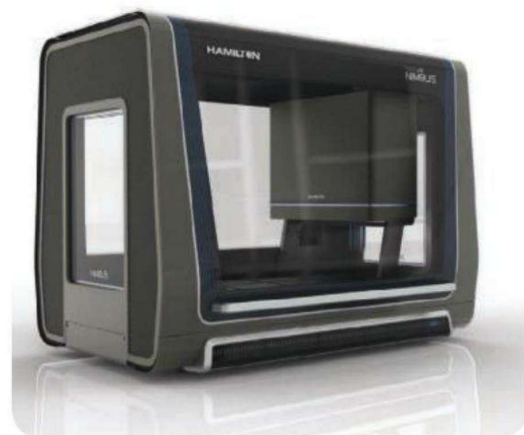
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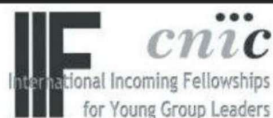
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The positions will be subject to Post-doctoral contracts and require a PhD degree with or without post-doctoral experience. Candidates should be proficient with the relevant methods and technologies. Applications should be e-mailed to positions_semm@iit.it and should include a CV, the e-mail addresses of 3 referees and a short statement on professional skills and interests (1 page max). Candidates should indicate in the title to which of the above position(s) they are applying, and should ask three referees to send their reference letters directly to the same e-mail address. Selected candidates will be initially interviewed by Skype.

Application deadline: July 9, 2012

In order to comply with the Italian law (art. 23 of Privacy Law of the Italian Legislative Decree n. 196/03), we kindly ask every candidate to provide his/her consent to allow IIT to process his/her personal data. We inform you that the information you provided will be used solely for the purpose of assessing your professional profile to meet the requirements of Istituto Italiano di Tecnologia. Your data will be processed by Istituto Italiano di Tecnologia, with headquarters in Genoa, Via Morego, 30, acting as the Data Holder, using computer and paper based means, observing the rules on protection of personal data, including those relating to the security of data. Please also note that, pursuant to art. 7 of Legislative Decree 196/2003, you may exercise your rights at any time as a party concerned by contacting the Data Manager. The Italian Institute of Technology is an Equal Opportunity Employer that actively seeks diversity in the workforce.

SIU School of Medicine

Department of Medical Microbiology, Immunology, and Cell Biology (MMICB)

3 Tenure Track Positions: Immunology, Microbial Pathogenesis, Cancer Biology

Southern Illinois University's School of Medicine at Springfield invites applications for three tenure track faculty positions to join a supportive, interactive research environment within the MMICB (www.siumed.edu/mmi). Appointments will be at the rank of Assistant, Associate or Full Professor depending upon qualifications. The MMICB department has active research programs in areas including virology, neuroscience, reproductive immunology, developmental genetics, host responses to infection, and cancer biology with excellent records of extramural research funding from the NIH, DOD, etc. The Department's graduate program is part of the integrated Molecular Biology, Microbiology and Biochemistry Program (46 participating faculty members) and the department hosts ~40 M.S. and Ph.D. students from the program. The Department occupies new and renovated laboratory space and shares a CDC certified BSL3 laboratory with the IL Department of Public Health.

For all three positions, we seek energetic scientists demonstrating strong commitments to teaching and research, collaborative potential, and an ability to bring novel expertise to the institution. Appointments are State-supported with competitive salaries and start up packages with modern laboratory facilities. Successful applicants will be expected to establish and maintain an independent, externally funded research programs and will contribute to both medical student and graduate education. Excellent written and verbal communication skills are required. Appointment at Associate or Full Professor levels will require current and sustained records of extramural research funding. Eligible candidates must possess Ph.D. and/or M.D. degrees, and 3+ years of postdoctoral research training with strong publication records. We seek scientists with research interests, expertise and medical relevance in the following areas:

Immunology: molecular and cellular aspects of immune system function.

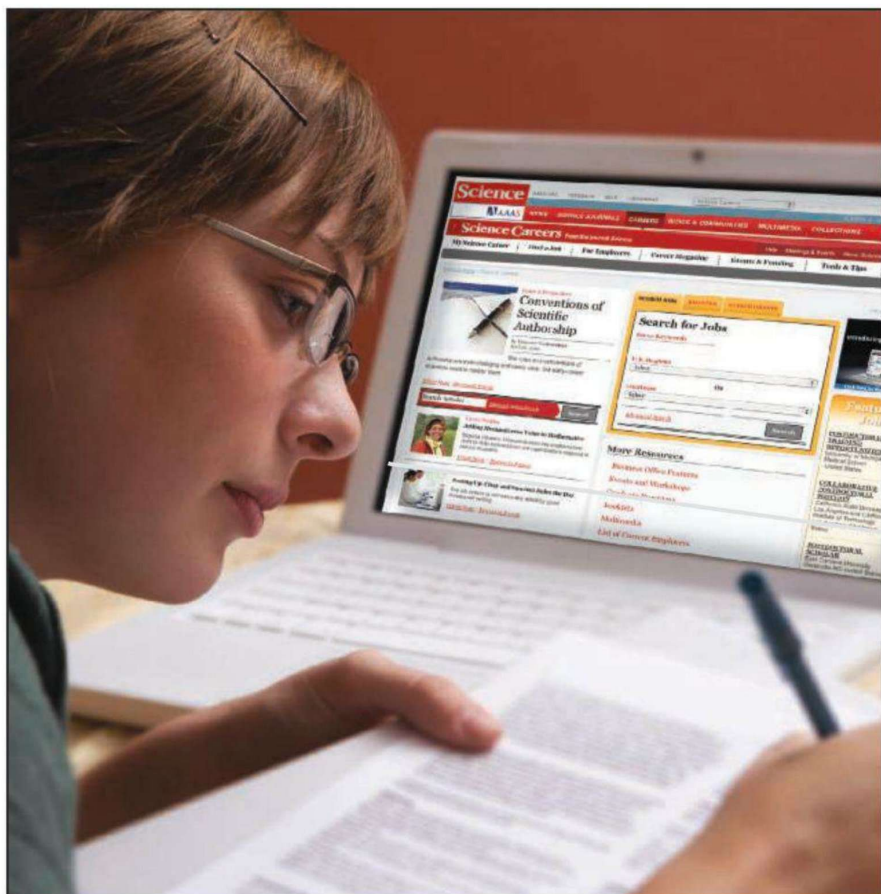
Microbial pathogenesis: molecular and cellular mechanisms of infectious disease or microbial-host interactions that control disease outcomes.

Cancer biology: basic and translational research to complement existing strengths in breast, prostate and colon cancer.

Applicants should submit a letter of interest addressing the position to which they are applying. Include a curriculum vitae with history of external funding, future research plans, statement of teaching interests and experience, and the names, addresses, telephone numbers and email addresses of at least three professional references in a single PDF to the email address below: **Dr. Donald S. Torry, Chair, MMICB Search Committee, c/o: tcasson@siumed.edu, P.O. Box 19626, Springfield, IL 62794-9626.**

Review of applications will begin immediately and continue until the positions are filled.

The Southern Illinois University School of Medicine is an equal opportunity, affirmative action employer. Background investigation required.



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Announcement of Job Vacancy Division on Earth and Life Studies Executive Director

The National Research Council is actively seeking an Executive Director for the Division on Earth and Life Studies. This is a rare and challenging opportunity for an executive professional with experience drawn from a broad range of related disciplines in the geosciences and life and environmental sciences.

The Executive Director is responsible for the intellectual, strategic, managerial, and financial leadership of the Division on Earth and Life Studies, which is one of the five divisions of the National Research Council (NRC). The Executive Director establishes current and long-range objectives for the division, manages all aspects of the division's activities and staff, works with individuals and organizations to secure financial support for the division's programs and projects, oversees the recruitment and support of volunteer advisers for the division's activities, and supports the institution's leadership in management and policy making.

The NRC operates under the charter of the National Academy of Sciences (NAS), along with the National Academy of Engineering (NAE) and the Institute of Medicine (IOM). The four organizations share a common mission of providing information and advice to the government and the public on matters involving science, engineering, technology and health. The NRC is the chief operating arm of the NAS and the NAE. It has approximately 1000 employees and an annual program budget of approximately \$300 million. More than 7000 volunteer advisers with scientific and technical expertise participate in its activities every year. The Division on Earth and Life Studies has approximately 120 employees and annual program expenditures of approximately \$30 million. The Executive Director reports to the Executive Officer of the NRC.

QUALIFICATIONS:

- Ph.D. in a related field or equivalent knowledge with at least six years of scholarship, research, and professional experience in a related field, at least three of which are in an executive capacity involving program development and leadership and the management and supervision of technical, budget and administrative staff.
- Experience in managing externally funded research involving multiple projects and sponsors.
- Experience working in complex organizations with science, engineering or health missions.
- Broad competence and intellectual interest in diverse fields among the earth and life sciences, including experience in research and scholarship, preferably including interdisciplinary work.
- Excellent written, oral, public speaking, interpersonal, and organizational skills with a proven ability to effectively interact with employees at all levels, volunteer advisers, sponsors, and others.

For more information about this challenging career opportunity and to apply, please visit our website at <http://national-academies.org>. Under Careers, search our Current Opportunities by Department – Div on Earth & Life Studies – **requisition number 120092-1**.

(Recruitment for this position will continue until an outstanding candidate is identified. It is expected that the candidate selected will begin employment as the DELS Executive Director no later than January 2013.)

EOE, M/F/D/V

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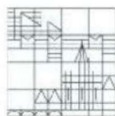
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The University of Konstanz belongs to the universities in Germany whose "Future concepts for top-class research at universities" is promoted within the framework of the Excellence Initiative of the German federal and state governments.

The *Zukunftskolleg* of the University of Konstanz is offering:

- A. up to fifteen ZIF Marie Curie 2-year Postdoctoral Fellowships in any discipline represented at the University of Konstanz (Salary Scale 13 TV-L)**
for researchers in the early stage of their career, so as to enable them to develop and implement individual and independent research projects. Fellowships will begin on March 1, 2013, and end on February 28, 2015.
Reference number 2012/110
- B. up to eight ZIF Marie Curie 5-year Research Fellowships in any discipline represented at the University of Konstanz (Salary Scale 14 TV-L)**
for the development and implementation of individual research projects. Fellowships will begin on March 1, 2013, and end on February 28, 2018.
Reference number 2012/111
- C. up to five 5-year Research Fellowships in any discipline represented at the University of Konstanz (Salary Scale 14 TV-L)**
for the development and implementation of individual research projects. Fellowships will begin on March 1, 2013, and end on February 28, 2018.
Reference number 2012/109

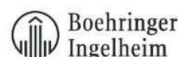
Applications should be submitted in English **before August 26, 2012**, to zukunftscolleg@uni-konstanz.de with the respective reference number in the subject line of the e-mail.



Contact: Anda Lohan LLM
e-mail: Anda.Lohan@uni-konstanz.de
phone: +49 (0) 75 31/88-48 21

Detailed information is available on our website:
<http://www.zukunftskolleg.uni-konstanz.de>

ANNOUNCEMENTS



Canadian Young Investigator Award

The recipient of the 2012 Boehringer Ingelheim Canadian Young Investigator Award in Biological Sciences is:



Sergio G. Peisajovich, Ph.D.

Assistant Professor, Department of Cell and Systems Biology
University of Toronto

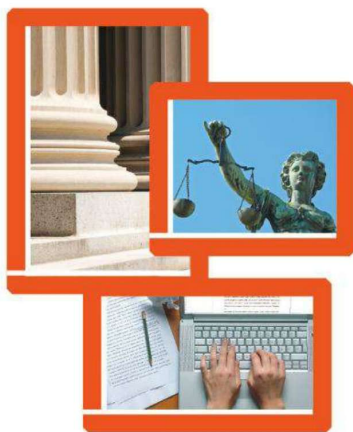
sergio.peisajovich@utoronto.ca

Dr. Peisajovich is an Assistant Professor in the Department of Cell and Systems Biology, at the University of Toronto. Dr. Peisajovich's research focuses on understanding how mutations, ranging from amino acid substitutions to gene duplication and recombination, affect the function and evolution of signaling networks. In particular, he is investigating how mutations in proteins belonging to MAPK-mediated networks drive tumor growth. In addition, he is interested in understanding how protein networks evolve, with the ultimate goal of using this knowledge to engineer novel cellular behaviors.

The R&D division of Boehringer Ingelheim (Canada) Ltd./Ltée is one of Canada's largest pharmaceutical research centres. One of our important corporate policies is to support and encourage basic research in Canadian universities. To this end, we have established the Boehringer Ingelheim Young Investigator Award in Biological Sciences. The award is made annually to a new faculty member conducting biological research in a Canadian university, and consists of an unrestricted three-year research grant.

Previous recipients

- 2011 Dr. Gavin Y. Oudit, University of Alberta
- 2010 Dr. Brian K. Coombes, McMaster University
- 2009 Dr. Anthony Gramolini, University of Toronto
- 2008 Dr. Marie Kmita, Université de Montréal



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AMC supports a diversified, smoke-free environment and is proud to be an Equal Opportunity/Affirmative Action Employer, encouraging women and minorities to apply. In support of a safe, drug-free environment, criminal background checks and drug testing are part of our hiring process.

DEPARTMENT CHAIR

Pharmacological and Physiological Science

Saint Louis University, a Catholic Jesuit institution dedicated to education, research, service, and health care, has started a national search for the next William Beaumont Professor and Chair of the Department of Pharmacological and Physiological Science (website: <http://medschool.slu.edu/pharmphys>). The successful applicant will have a Ph.D. or M.D. degree, a strong record of academic achievement in mechanistic aspects of cellular and molecular biology, a solid level of extramural research funding, experience in both graduate student and medical student education, and enthusiasm for mentoring faculty. Interested candidates should submit a cover letter, curriculum vitae, and a description of their leadership vision to website: <http://jobs.slu.edu> (Req. ID# 20120062). Letters of nomination may be sent electronically to **Enrico Di Cera, M.D.**, Chair of the Search Committee (e-mail: enrico@slu.edu).

Saint Louis University is an Affirmative Action/Equal Opportunity Employer, and encourages nominations and applications of women and minorities.

The Division of Hematology in the Department of Medicine at the University of Washington (UW) is recruiting an established molecular, genomics, or epigenomics investigator to lead a translational research program in normal or neoplastic hematopoiesis. M.D. or equivalent, and a strong record of research accomplishment and funding. Appointment to the full-time faculty at **ASSOCIATE or FULL PROFESSOR** level, tenure may be a consideration commensurate with qualifications. To be eligible for University sponsorship for an H-1B visa, graduates of foreign (non-U.S.) medical schools must show successful completion of all three steps of the U.S. Medical Licensing Exam (USMLE), or equivalent as determined by the Secretary of Health and Human Services. UW faculty engage in teaching, research, and service.

Electronically send curriculum vitae and statement of research interests to **Thalia Papayannopoulou**, Search Committee Chair, UW-Hematology, e-mail: thalp@uw.edu. Position open until filled.

The UW is building a culturally diverse faculty and strongly encourages applications from women and minority candidates. The UW is an Equal Opportunity/Affirmative Action Employer.

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POSITIONS OPEN



RESEARCH ASSISTANT PROFESSOR Department of Critical Care Medicine University Of Pittsburgh School of Medicine

The Department of Critical Care Medicine at the University of Pittsburgh School of Medicine seeks applications for Research Assistant Professor in Critical Care Medicine. The faculty member will be based in the Safar Center for Resuscitation Research and will focus on investigations germane to pathobiology and treatment in resuscitation, neurotrauma, neurocritical care and other aspects of critical care medicine. The selected individual will be required to plan, direct, and execute complex studies in resuscitation science. Applicants must hold a Doctorate in a relevant area of research, have postdoctoral experience, and have a track record of investigation and publication in neuroprotection/cytoprotection or cellular/molecular neurobiology including in vitro and in vivo investigations. A record of successful extramural funding is desirable.

Review of applications will begin on July 1, 2012 and will continue until the position is filled. Applicants should submit a statement of interest (up to three pages), curriculum vitae, and contact information for three references to: **Patrick M. Kochanek, M.D.**, MCCM, Director, Safar Center for Resuscitation Research, Professor and Vice Chairman, Department of Critical Care Medicine, University of Pittsburgh School of Medicine, 3434 Fifth Avenue - Room 100, Hill Building, Pittsburgh, PA 15260; telephone: 412-383-1900; fax: 412-624-0943; e-mail: kochanekpm@ccm.upmc.edu.

The University of Pittsburgh is an Affirmative Action/Equal Opportunity Employer.

ASSISTANT PROFESSOR of Stream Ecology

The University of Maine's School of Biology and Ecology invites applications for a tenure-track position (50% research/50% teaching) in stream ecology. The successful candidate will develop an internationally recognized research program with a primary focus on lotic systems and macroinvertebrates. Research should be relevant to Maine and the northeastern U.S., and may include (not limited to) ecosystem ecology, benthic community ecology, aquatic entomology, or system responses to human influences. We are especially interested in applicants who will complement the University's current strengths in aquatic ecology and foster integrative approaches to fundamental and applied problems. Teaching responsibilities will include undergraduate courses in stream/river ecology and aquatic entomology, along with periodic lecture or seminar contributions to the undergraduate ecology offerings. The faculty member is expected to advise undergraduates, train graduate students, and have a productive and extramurally supported research program. A Ph.D. is required in a relevant area of biological or environmental sciences, along with a background in lotic systems and macroinvertebrates. Further position information and important application instructions are available at website: <http://jobs.umaine.edu/blog/2012/05/19/assistant-professor-of-stream-ecology/>. Review of applications will begin September 7, 2012 and will continue until the position is filled. Incomplete applications cannot be considered. Appropriate background checks will be required. The University of Maine is an Equal Employment Opportunity/Affirmative Action Employer.

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LOCATION: Jackson Park Health Club
ARTICLE: *An Electronic Second Skin*
DATE: Sep 21, 7:43am

LOCATION: University Faculty Lounge
ARTICLE: *The Visual Impact of Gossip*
DATE: Sep 21, 4:22pm

LOCATION: Gyro King
ARTICLE: *Cavemen Craved Carbs, Too*
DATE: Sep 21, 1:13pm

LOCATION: Hemlock Bar
ARTICLE: *Quantum Simulation of Frustrated Classical Magnetism in Triangular Optical Lattices*
DATE: Sep 21, 9:21pm

LOCATION: Bed
ARTICLE: *Consciousness: What, How and Why*
DATE: Sep 21, 10:56pm



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